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FACULTY OF MEDICINE AND HEALTH SCIENCES

THE AIRWAY EPITHELIUM AS ORCHESTRATOR OF POLLUTANT-AGGRAVATED AIRWAY DISEASES

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ABBREVIATIONS

AHR	Airway Hyperresponsiveness
BALF	Bronchoalveolar Lavage Fluid
COPD	Chronic Obstructive Pulmonary Disease
ChemR23	Chemerin Receptor 23
DC	Dendritic Cell
DEPs	Diesel Exhaust Particles
ELISA	Enzyme-Linked ImmunoSorbent Assay
FEV_1	Forced Expiratory Volume in 1 second
FVC	Forced Vital Capacity
GATA-3	Trans-acting T-cell-specific transcription factor
GINA	Global Initiative for Asthma
GM-CSF	Granulocyte-Monoctye Colony Stimulating Factor
GOLD	Global Initiative for Obstructive Lung Diseases
GWAS	Genome Wide Association Studies
HBEC	Human Bronchial Epithelial cells
HDM	House Dust Mite
HRP	Horseradish Peroxidase
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate Lymphoid Cells
IN	Intranasal
IP	Intraperitoneal
IRAK	IL-1R-associated kinase
IV	Intravenously
KO	Knockout
MAPK	Mitogen-activated protein kinases

MHC	Major Histocompatibility Complex
MLN	Mediastinal Lymph Nodes
MYD88	Myeloid differentiation primary response protein 88
NF-κB	Nuclear factor-κB
OD	Optical Density
OVA	Ovalbumin
pDC	Plasmacytoid DC
PM	Particulate Matter
PRR	Pattern recognition receptors
RAG	Recombination-Activating Gene
RORα	RAR-related Orphan Receptor α
ROS	Reactive Oxygen Species
r-sST2	Recombinant soluble ST2
qRT-PCR	quantitative Real Time-Polymerase Chain Reaction
T_H cell	T helper cell
TLR	Toll-like receptor
TRAF	Tumour necrosis factor receptor-associated factor
TSLP	Thymic Stromal LymphoPoietin
UFPM	Ultrafine particulate matter
WHO	World Health Organization
WT	Wild-Type
ZO-1	Zonula Occludens-1

PART I: INTRODUCTION

CHAPTER 1: CHRONIC AIRWAY DISEASES

1.1 Introduction

Worldwide, millions of people suffer from chronic respiratory diseases that affect the airways and structural components of the lung. The most common chronic airway diseases are asthma and chronic obstructive pulmonary disease (COPD). They have major adverse effects on the quality of life and are one of the major grounds for consulting healthcare centres. Currently, these diseases cannot be cured, however various treatment options are available to relieve the patient's symptoms and improve their quality of life [1].

1.2 Asthma

Asthma is a chronic respiratory disease of the conducting airways, affecting 334 million people worldwide [2]. According to the Global Initiative for Asthma (GINA), asthma is described as "*a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation*" [3, 4]. The airway of asthmatics is characterized by several pathological changes, including inflammatory cell infiltration, goblet cell metaplasia, airway hyperresponsiveness (AHR), airway wall remodeling and reversible airway obstruction (Figure 1) [5].

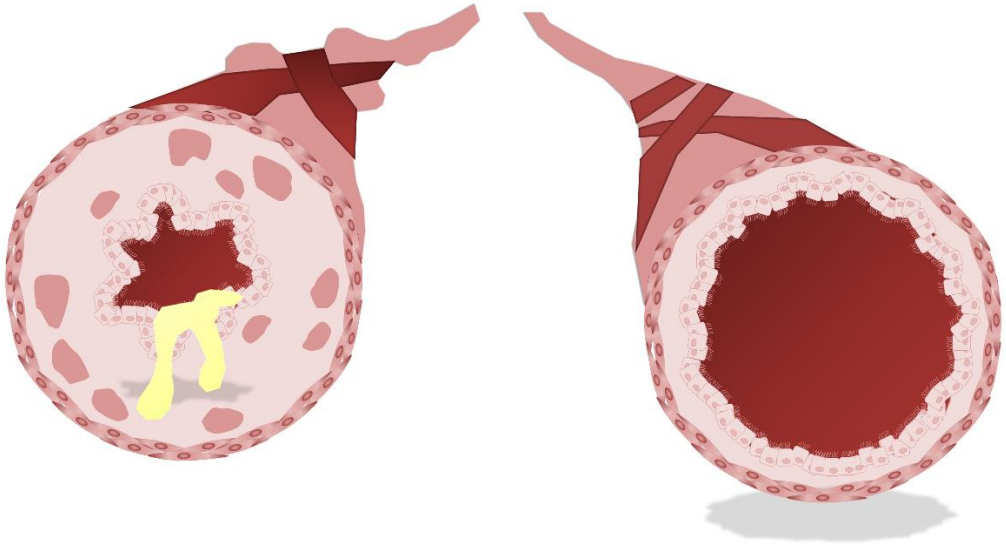


Figure 1: Bronchial tube of a healthy person (right) and asthmatic patient (left), characterized by inflammatory cell infiltration, excessive mucus production, smooth muscle hypertrophy and airway lumen narrowing.

Asthma can be diagnosed based on the patient's characteristic pattern of respiratory symptoms that vary over time and intensity and the presence of a bronchial hyperresponsiveness and variable airflow limitation. The airflow obstruction is measured with spirometry and defined as the ratio of the forced expiratory volume in one second (FEV_1) to the forced vital capacity (FVC), the tiffeneau index. FEV_1 is the maximum volume of air that can be forcedly exhaled in one second after complete inspiration, whereas FVC is defined as the maximum volume of air that can be forcedly exhaled. In asthmatics, the airflow limitation is largely reversible which means that their FEV_1 should improve rapidly after inhalation of a bronchodilator during bronchial provocation tests [3].

Over the years it became clear that asthma is a complex and heterogeneous disease with different underlying disease processes. More specifically, multiple phenotypes can be distinguished based on the patient's clinical characteristics and inflammatory profile (Figure 2) [6]. The best characterized phenotype is (early-onset) allergic asthma, defined by the presence of allergen-specific immunoglobulin E (IgE) in serum and/or a positive skin prick test to common allergens, such as house dust mite (HDM), in association with type 2 mediated immune responses. Other phenotypes are i.e. late-onset eosinophilic asthma and smoking-associated neutrophilic asthma [6, 7].

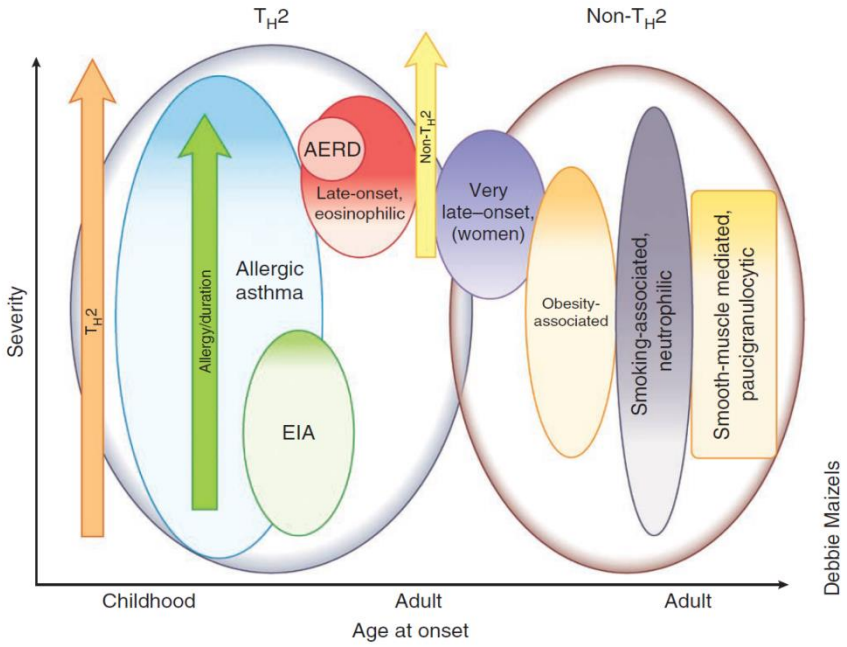


Figure 2: Emerging asthma phenotypes. Multiple asthmatic phenotypes can be distinguished based on the type of inflammation, severity and age at onset. EIA: Exercise-induced asthma; AERD: Aspirin exacerbated respiratory disease. Reprinted by permission from Macmillan Publishers Ltd: [Nature Medicine] (Wenzel SE. Nat Med 2012;18: 716-25[6]). Copyright (2012).

1.2.1 Pathogenesis of allergic asthma

The lung is continuously exposed to different environmental factors (allergens, microbes, viruses and pollutants). In healthy individuals, specialized regulatory mechanisms are present to achieve immune tolerance and maintain homeostasis against self-antigens or foreign matter [8, 9]. Failure of these tolerance mechanisms can however lead to excessive immune responses and development of inflammatory diseases such as asthma (Figure 3).

Most asthmatics become sensitized to common aeroallergens, especially those derived from house dust mite (HDM), animal dander, fungi and pollen [10]. **Sensitization** to an allergen is defined as the process between the first encounter and the formation of allergen-specific IgE complexes while no allergic symptoms are present [11]. Initially, allergens are taken up by specialized antigen presenting cells, the dendritic cells (DCs). These DCs reside near the epithelium and can sample the airway lumen for danger signals derived from the allergen itself and/or the airway epithelium (see chapter 3). The DCs process the allergen into small peptides and traffick to the lymph nodes, where antigen presentation to naïve $CD4^+$ T cells occurs. During their migration, the DCs mature by expressing an array of co-stimulatory molecules (i.e. CD80 and CD86) and high levels of MHCII molecules to induce proliferation and differentiation of allergen-specific T helper 2 (T_H2) cells. These T_H2 cells will then in turn secrete IL-4 and IL-13, type 2 cytokines that are both capable of stimulating an immunoglobulin switch of B cells from IgM to IgE. This allergen-specific IgE can then bind with high affinity receptors (FcεRI) expressed on a variety of different immune cells, such as mast cells, basophils and eosinophils [7, 10, 12].

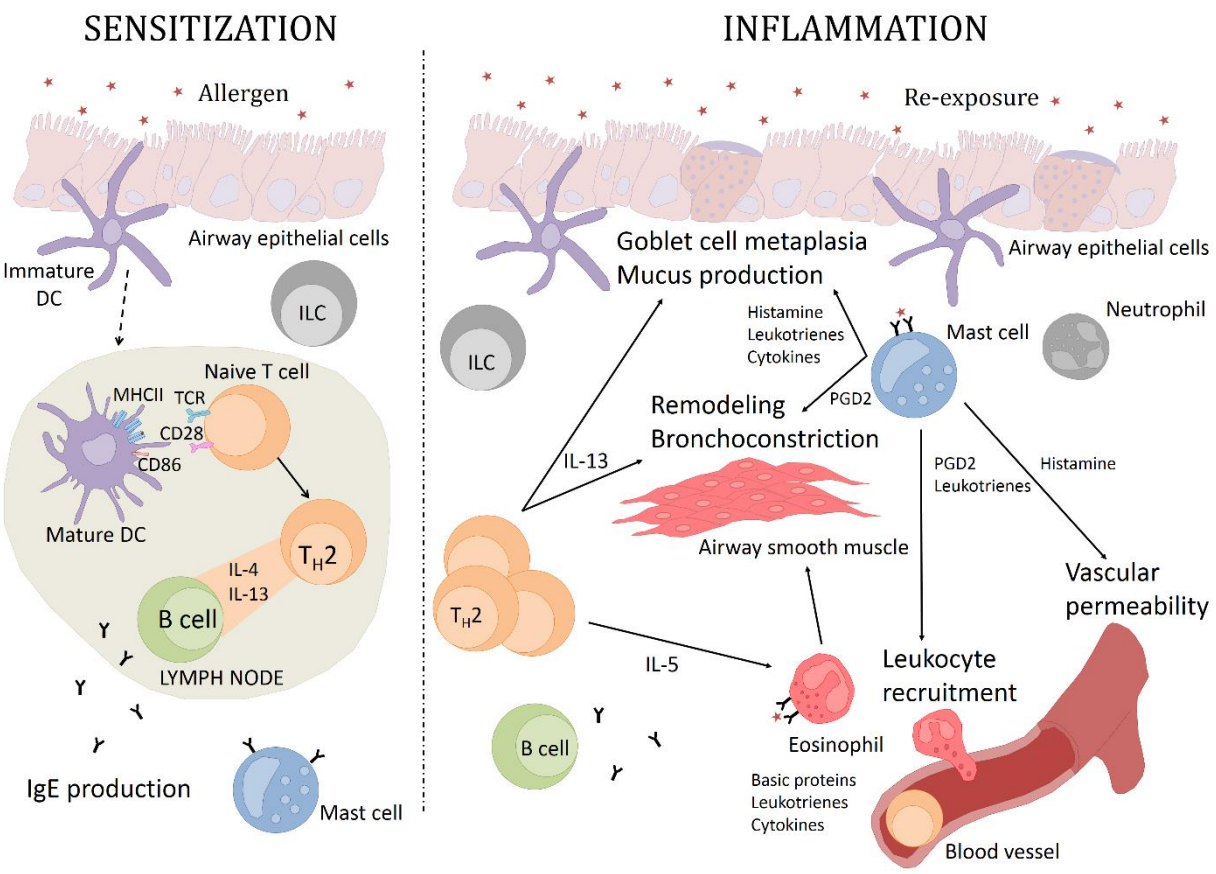


Figure 3: Pathogenesis of allergic asthma. DCs mature to competent antigen presenting cells in response to various danger signals, such as allergens. Allergen-loaded DCs migrate to the lymph nodes and interact with naïve T cells to drive T_H2 polarization. Type 2 cytokine production of T_H2 cell stimulate B cells to produce IgE which will bind with FcεRI expressing cells. Allergen re-exposure will lead to an inflammatory process wherein inflammatory cells are recruited to the airways and cell activation occurs, resulting in the release of specific mediators contributing to the characteristic features of asthma.

Once sensitization has occurred, allergen **re-exposure** will induce a local infiltration of allergen-specific T_H2 cells which will produce type 2 cytokines, i.e. IL-5 and IL-13, contributing to the hallmarks of asthma. Moreover, the recruitment of various inflammatory cells, such as mast cells and eosinophils, to the airways will occur. Crosslinking of allergen-specific IgE molecules on mast cells for instance will lead to their activation and release of preformed mediators containing histamine, lipid mediators (leukotrienes and prostaglandins), cytokines, chemokines and growth factors, responsible for bronchoconstriction, vasodilation, increased vascular permeability, excessive mucus production and ongoing inflammatory cell recruitment. Furthermore, an infiltration of eosinophils into the lung will occur, mediated by IL-5, a type 2 cytokine which is responsible for the proliferation, activation and survival of eosinophils. These prominent cells are a rich source of inflammatory proteins, including major basic protein, leukotrienes and cytokines, contributing to tissue damage, bronchoconstriction and airway wall remodeling [5, 10, 13].

Besides T_H2 cells, also innate lymphoid cells (ILC) have emerged as important contributors in the pathogenesis of asthma. Their role and mechanism of action will be extensively discussed in chapter 4.

1.2.2 Risk factors

Although the precise causes of asthma remain unclear, studies suggest that several risk factors can influence the development and phenotype of asthma, categorized as host factors and environmental factors (Figure 4).

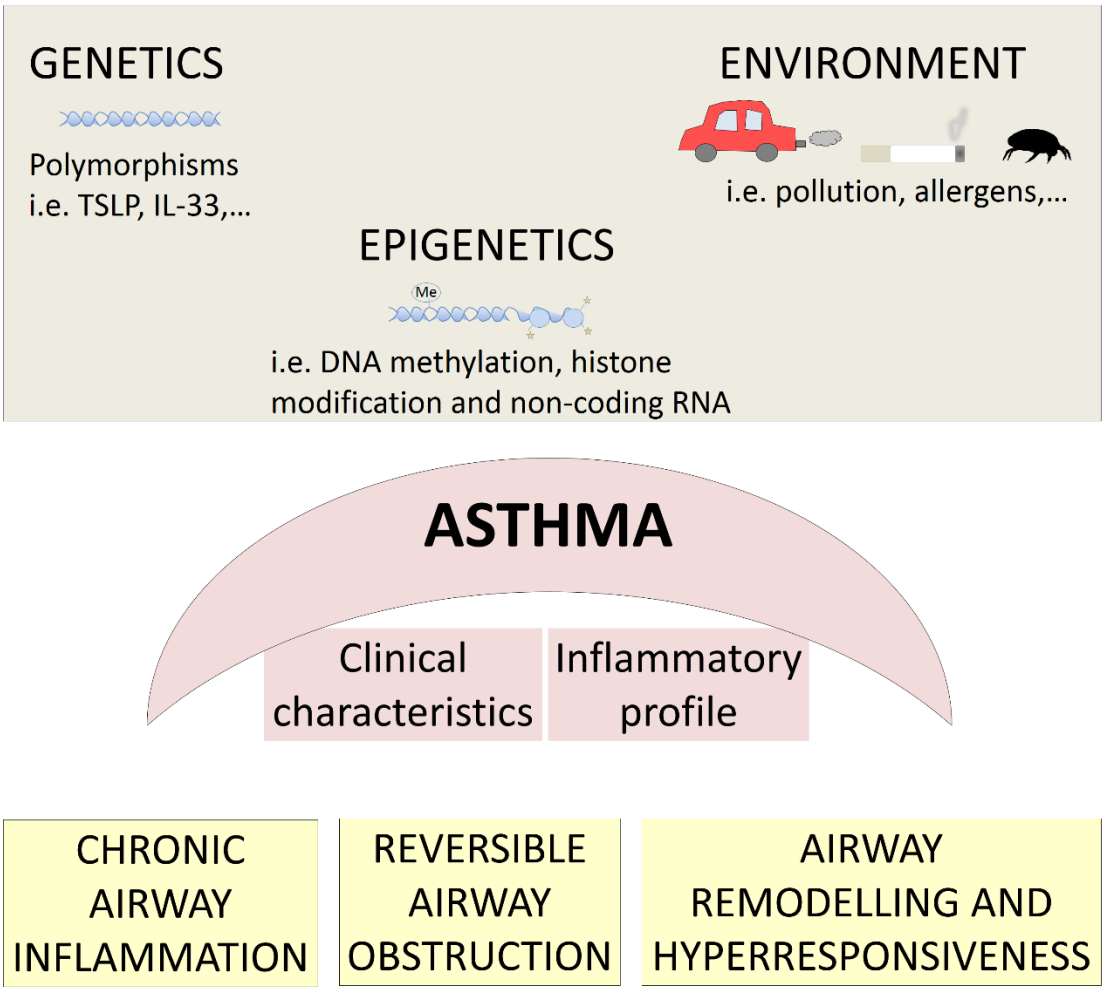


Figure 4: Risk factors contributing to the development and phenotype of asthma. The development of asthma is strongly regulated by heritable components (genetics and epigenetic mechanisms) and exposure to environmental factors. Asthma is a heterogeneous disease wherein multiple phenotypes can be distinguished based on clinical characteristics and the inflammatory profile. The disease is characterized by a chronic airway inflammation, reversible airway obstruction, airway wall remodeling and airway hyperresponsiveness.

Genetics have a strong influence on the development of asthma, meaning that individuals can be genetically predisposed to be protected or susceptible to develop asthma. Twin studies provided convincing evidence that asthma is a heritable disease, wherein approximately 60-80% of asthma susceptibility is determined by genetic factors such as nucleotide variants [14, 15]. Such genetic polymorphisms, related to asthma susceptibility, have already been identified by the development of genome wide association studies (GWAS). As an example, polymorphic changes in the *TSLP* and *IL-33* gene have brought attention to the airway epithelium as a key factor in the development of asthma (see chapter 3) [16, 17]. Despite the identification of such disease-associated loci, only a small proportion of heritability can be explained by genetic variants [18, 19].

The rapid increase in the incidence of asthma over the last decades and the discordance of developing asthma that is observed in twin studies, point to the fact that the etiology of asthma is also influenced by environmental factors. It is for instance demonstrated that also complex gene-by-environment interactions occur, mediated by epigenetic mechanisms. **Epigenetics** can be defined as mechanisms that regulate gene expression without altering the underlying DNA sequence itself. Epigenetic changes include DNA methylation, histone modifications and non-coding RNAs [19-21]. Currently, several environmental factors, such as pollutants, infections and allergens, can exert epigenetic changes, contributing to the pathogenesis of asthma [21, 22].

An enhanced susceptibility to the onset and exacerbation of asthma can be further attributed to **environmental factors**, including exposure to air pollution (see chapter 2) and allergens (i.e. HDM, pollen, animal dander) which can induce and facilitate inflammatory immune responses [23]. The most important source of perennial indoor allergens is **HDM**, a complex organism found in 70% of the bedsheets and mattresses where they feed on human skin scales. Mite allergy is highly prevalent in the world, with around 50-85% of asthmatics that are sensitized to HDM allergens [24, 25]. The most common mite species are *Dermatophagoides pteronyssinus* and *D. farinae* [26]. So far, over 20 different protein families have been identified as HDM allergens (Table 1), which are present in high amounts in the mite bodies and fecal pellets [24, 25]. Among these different HDM allergens, proteases are highly abundant and being held responsible for the inflammatory properties of HDM [27, 28]. Especially group 1 and 2 allergens have been identified as major allergens as 80% of allergic patients are sensitized to Derp1 and Derp2. Derp1 belongs to the cysteine protease family [24, 29] and has been shown to target tight junction proteins, increasing the permeability of the airway epithelium (see chapter 3) [27]. The second major allergen is Derp2, a lipid binding protein that can activate innate immune responses through toll-like receptor 4 (TLR4) signaling [30]. Moreover, also lipopolysaccharide (LPS) and β-glycans can be detected in the HDM extract [25].

Table 1: Characterized allergens of *Dermatophagoides pteronyssinus*. All allergens listed are officially listed in the WHO/IUIS allergen database **[31]**.

Allergen group	Allergen	Protein family
1	Derp1	Cysteine protease
2	Derp2	NPC2 family
3	Derp3	Trypsin
4	Derp4	α-Amylase
5	Derp5	-
6	Derp6	Chymotrypsin
7	Derp7	-
8	Derp8	Gluthathion-S-Transferase
9	Derp9	Serine protease
10	Derp10	Thopomyosin
11	Derp11	Paramyosin
12	Derp12	-
13	Derp13	Fatty acid-binding protein
14	Derp14	Apolipophorin
15	Derp15	Chitinase-like protein
18	Derp18	Chitin-binding protein
20	Derp20	Arginine kinase
21	Derp21	-
23	Derp23	Peritrophin-like protein domain
24	Derp24	biquinol-cytochrome c reductase binding protein
26	Derp36	-

Besides the well-known association between environmental exposures (i.e. pollution and allergens) and increased respiratory symptoms, it was demonstrated that microbial exposures can protect against the development of atopic diseases such as asthma, the so called **"hygiene hypothesis"** [32]. This hypothesis was originally raised by David Strachan who observed an inverse relationship between the number of siblings and the rate of allergic diseases [33]. Furthermore, rural living conditions (growing up on farms) have been associated with a reduced risk of asthma development. A recent study for instance demonstrated a lower prevalence of asthma in the Amish community that practice traditional farming (high microbial exposure) compared to the Hutterites that implement industrial farming [34]. Recently, the protein A20, located in lung epithelium, was identified as an important determinant on how high endotoxin

levels or farm dust could protect mice from developing HDM-induced allergic airway inflammation [35].

1.2.3 Treatment

Despite the fact that asthma is not curable, there are several treatment options to improve the patient's symptoms and quality of life. The most common treatment is focused on the combination of relieving airway obstruction with short- or long-acting β2-adrenergic receptor agonists and reducing airway inflammation by using inhaled corticosteroids [3, 36, 37]. However, for patients with severe asthma that require high doses of inhaled or even oral corticosteroids, there is an urgent need for more targeted approaches [38, 39]. Identifying the different asthmatic phenotypes [6], is becoming more important in asthma treatment, that is evolving to precision or personalized medicine [40]. To date, there are few approved biologics available as add-on therapy in the treatment of asthma that are currently reserved for poor-controlled patients with a specific asthma phenotype. Omalizumab is a humanized monoclonal IgE-targeting antibody recommended for allergic patients. Omaluzimab forms complexes with circulating IgE, preventing its interaction with FcεRI expressed on mast cells, eosinophils, dendritic cells and basophils, which are important cells in the pathogenesis of asthma [37, 41]. Adding omalizumab to standard therapy in severe allergic asthmatics led to reduced asthma exacerbations and corticosteroid-sparing effects [42]. Mepolizumab and reslizumab, humanized monoclonal IL-5 targeting antibodies, reserved for patients with a persistent eosinophilic inflammation, will target IL-5, an important type 2 cytokine that promotes the growth, maturation and activation of eosinophils [37, 41, 43]. Treatment with such anti-IL-5 monoclonal antibodies has led to a reduction in the exacerbation rate and/or reduced use of corticosteroids in asthmatic patients with elevated eosinophils in the blood or sputum [44-46]. Interestingly, a post hoc analysis revealed that especially patients with late-onset eosinophilic asthma greatly improved with reslizumab as add-on therapy compared to early-onset asthma [47]. Currently, promising results are also obtained with anti-IL-4Rα (dupilumab) and anti-IL-5R (benralizumab) monoclonal antibodies as add-on treatment for persistent asthmatics [48, 49]. Moreover, a diversity of new molecules (i.e. monoclonal antibodies directed against epithelial-derived cytokines) are tested for safety and efficacy in clinical trials [43]. It was for instance recently demonstrated that severe uncontrolled asthmatics who received a monoclonal anti-TSLP antibody (Tezepelumab) had significant fewer asthma exacerbations compared to placebo [50].

1.3 Chronic Obstructive Pulmonary Disease (COPD)

COPD is a respiratory disease of the smaller airways and parenchyma, affecting 210 million people worldwide. In 2015, it was estimated that about 3 million deaths were attributable to COPD, making it the fourth leading cause of death [51]. COPD is defined by the global initiative for chronic obstructive lung disease (GOLD) as *"a common, preventable and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases"*. Patients who suffer from COPD typically present with symptoms as progressive and persistent dyspnea, chronic cough and sputum production, often accompanied by systemic effects and comorbidities [52].

The golden standard to diagnose COPD is by performing spirometry in symptomatic patients that have a history of exposure to risk factors. By assessing the severity of the airflow limitation, which is based on the FEV_1 post-bronchodilator value, COPD is classified into 4 different stages: mild ($FEV_1 \geq 80\%$; GOLD I), moderate ($50\ \% \leq FEV_1 \leq 80\ \%$; GOLD II), severe ($30\ \% \leq FEV_1 \leq 50\ \%$; GOLD III) and very severe ($FEV_1 < 30\ \%$; GOLD IV) COPD. Recently, a new classification method was proposed, the ABCD assessment tool, that takes into account the evaluation of symptoms and the history of exacerbations on top of the severity of airflow limitation [52].

The main risk factor for developing COPD is tobacco smoking. Other risk factors include indoor and outdoor air pollution and occupational exposures to dusts and fumes. However, considering that only 20% of smokers develop COPD, also genetic factors will predispose individuals to develop COPD. Typical pathophysiological hallmarks of COPD include a chronic airway inflammation, lymphoid neogenesis, emphysema and airway wall remodeling [53].

Although COPD cannot be cured, it is currently managed by controlling symptoms and reducing exacerbations with bronchodilators and inhaled corticosteroids [52]. On top, smoke cessation is key to influence the natural history of COPD and specifically slowdown the accelerated decline in lung function [54].

CHAPTER 2: AIR POLLUTION

2.1 Introduction

Air pollution is a major cause of environmental health problems, leading to increased morbidity and mortality. It is estimated that each year approximately 3 million deaths are solely attributed to ambient (outdoor) air pollution [55]. It is a complex mixture including particulate matter (PM), chemicals, such as ozone, and biological substances. Although all components of air pollution are harmful for human health, severe effects have been attributed to PM since they can carry a broad range of toxic substances into the respiratory tract [56]. Ambient PM consists of solid and liquid particles that are generated as a result of natural processes as well as human activity, such as industry, cigarette smoke and traffic. The particulates differ in their chemical composition and are divided into 3 categories based on their aerodynamic diameter: coarse PM (< 10µm, PM_{10}), fine PM (< 2.5µm, $PM_{2.5}$) and ultrafine PM (UFPM) (< 0.1µm, $PM_{0.1}$).

The ability of ambient PM to induce lung injury and inflammation is determined by several key characteristics, such as particle size, surface area and chemical composition [57-59] (please also see addendum, review). Importantly, the most health-damaging effects have been attributed to UFPM, since they can deposit deep in the pulmonary tissue, into the smaller airways and alveoli, and some will even translocate to the systemic circulation, causing extra-pulmonary effects (Figure 5) [60]. Moreover, as the particle size decreases, its surface area increases, allowing a greater proportion of metals and organic carbons at the particle's surface, which are implicated in pro-oxidant and pro-inflammatory effects in the respiratory system [57, 61-63].

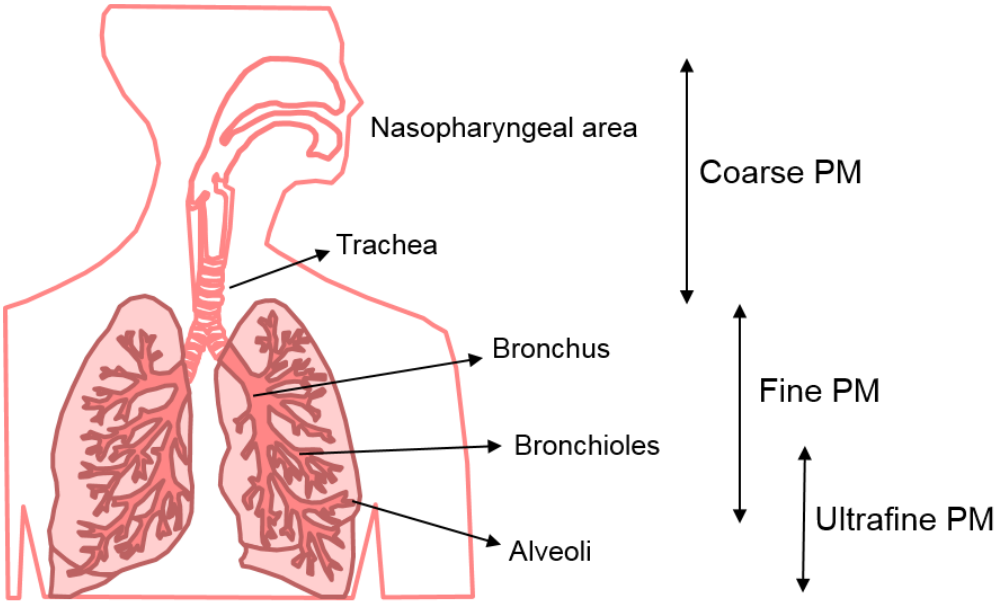


Figure 5: Deposition particulate matter. Coarse PM (PM_{10}) deposits mainly in the nose and large conducting airways, whereas the deposition of $PM_{2.5}$ is located throughout the respiratory tract, particularly in the small airways and alveoli. The ultrafine PM fraction $PM_{0.1}$ will further deposit in the pulmonary tissue and some will even translocate from the alveoli to the pulmonary circulation.

A major component of (ultra)fine traffic-related ambient PM are diesel exhaust particles (DEPs). These particles are mainly derived from vehicles during the combustion of diesel fuels. DEPs consist of a carbon core which can adsorb several organic compounds, such as polycyclic aromatic hydrocarbons (PAHs) and quinones. Additional, traces of sulphates, nitrates and metals can be observed [64, 65].

2.2 Impact on human health

About 90% of the world's population breathe air that is not conform to the WHO guidelines (i.e. annual mean values of 10 $\mu g/m^3$ for $PM_{2.5}$), resulting in a serious disease burden attributable to ambient air pollution [55]. In 2015, exposure to 10 $\mu g/m^3$ $PM_{2.5}$ was exceeded at 75% of the European measuring stations (Figure 6). Although the air quality in Europe is slowly improving, the European Environment Agency (EEA) states that air pollution still has a significant impact on Europeans' health. Air pollution is still associated with more than 500.000 premature deaths per year in Europe, making it the leading environmental cause of premature deaths. Four out of five premature deaths were directly linked with fine PM [66]. Alarmingly, it was recently demonstrated that also pollution levels below the current national standards are associated with an increased risk of death [67].

Several epidemiological studies already demonstrated a positive correlation between air pollution and multiple chronic diseases, ranging from respiratory diseases, such as asthma, COPD and lung cancer, to cardiovascular illness and all-cause mortality [60, 68-70]. To date, cardiovascular diseases are the leading causes of death worldwide [71]. Notably, in 2016, > 2 million premature cardiovascular deaths out of 17 million were attributed to ambient air pollution [72]. Specific causal associations were found between PM exposure and myocardial infarction, hypertension, congestive heart failure, arrhythmias and cardiovascular mortality [73]. Besides cardiovascular events, exposure to air pollution was also associated with increased cancer incidence, especially lung cancer [74]. In the ESCAPE study, a large European cohort, long-term exposure to PM air pollution contributed to the lung cancer incidence in Europe [75]. It was therefore not surprising that in 2013, the International Agency for Research on Cancer (IARC) reported ambient air pollution, as well as PM as a separate component, as group 1 human carcinogens [74].

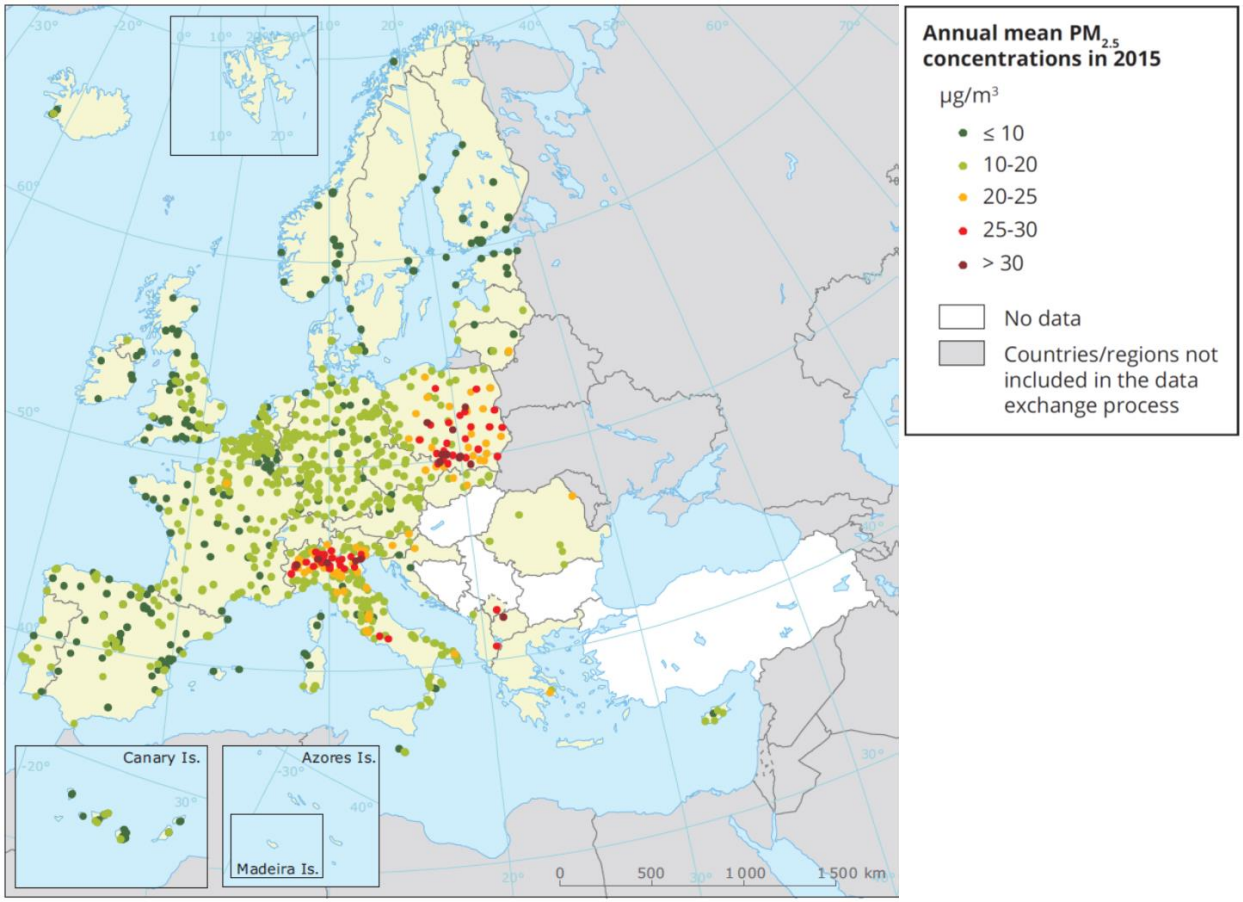


Figure 6: The annual mean $PM_{2.5}$ concentration in 2015 [66].

While the WHO estimates that 18% of air pollution-related premature deaths are due to COPD and acute lower respiratory infections [76], various epidemiological studies indicated that exposure to ambient PM and DEPs can also contribute to the onset as well as exacerbation of both childhood and adult-onset asthma [60, 77-81]. Living near major roads was for instance associated with significantly more respiratory symptoms and adverse effects on lung function in children and adults [70, 82-85]. Interestingly, the first 8 years of life can be considered as a susceptible period, as traffic-related PM exposure during that period was associated with an impaired lung function growth [86]. Furthermore, when asthmatic individuals were exposed to high levels of particulate pollution, they were more likely to experience worsened symptoms and impaired lung function, leading to uncontrolled asthma and hospitalizations [79, 81, 87]. Asthmatics walking on a polluted street in London had a higher airway inflammation and reduction in lung function compared to those walking in a low traffic environment [88]. Although PM and DEPs are without a doubt important determinants in the onset and aggravation of asthma, specific mechanisms are incompletely known.

2.3 Diesel exhaust particles and acute immune responses

It has been well accepted that DEPs can cause several adverse effects on their own, inducing acute inflammatory responses in the nose, throat and lungs [23]. Both human and animal studies have shown that exposure to DEPs is associated with increased proinflammatory cytokine/chemokine release, generation of reactive oxygen species (ROS), inflammatory cell influx in the lung, airway resistance and remodeling [89-93] (see addendum, review). The underlying mechanisms that contribute to these DEPs-induced acute immune responses need however to be further elucidated. It was already demonstrated that DEPs modulate DC biology, influence its recruitment, maturation and migration towards the mediastinal lymph nodes (MLN), resulting in Th2 polarization. Influencing DC function could therefore be an important mechanism by which DEPs contribute to asthma [94, 95].

2.4 Diesel exhaust particles and allergen-induced immune responses

In addition to these acute effects, DEPs can facilitate allergen-induced immune responses, contributing to the pathogenesis of asthma [23]. In murine models, DEPs can promote the development of an allergic airway inflammation towards for instance house dust mite (HDM). More specifically, combined DEPs+HDM exposure increased eosinophilic airway inflammation, type 2 cytokine production, allergen-specific immunoglobulins, AHR and remodelling compared to sole DEPs or HDM exposure [96-98]. Controlled human exposure studies further confirmed that allergen-specific responses, such as airway eosinophilia, type 2 cytokine expression and allergen-specific IgE, can be augmented when the individuals were exposed to both DEPs and an (neo)allergen [99-101]. Whereas for the moment predominantly descriptive studies have been performed, the underlying cellular and molecular mechanisms remain to be investigated. In this dissertation, mechanisms contributing to the enhancing effects of DEPs on the allergic airway inflammation are assessed.

CHAPTER 3: THE AIRWAY EPITHELIUM

3.1 Introduction

The airway epithelium is an important line of defence to a wide variety of environmental factors, including allergens and pollutants (Figure 7) [102]. A first crucial defence mechanism that protects the lung from foreign particles is the mucociliary apparatus located on top of the epithelial surface. This mucus layer traps inhaled substances which are transported out of the lung by ciliary beating and coughing, a process called **mucociliary clearance**. In asthmatics however, excessive mucus production and ciliary dysfunction leads to an impaired clearance, resulting in plug formation and airflow obstruction [103, 104]. Moreover, in PM-exposed human bronchial epithelial cells (HBEC) the ciliary beat frequency was progressively attenuated [105], suggestive for a delayed clearance of such particles out of the lung.

The integrity of the epithelial barrier is further regulated by a coordinated expression of apical tight junctions (claudins, occludin, zonula occludens (ZO)-1, 2, 3), underlying adherens junctions (E-cadherin, β-catenin) and desmosomes [102, 104, 106]. Cleavage of such **epithelial junctional complexes** and disruption of the barrier structures can be mediated directly by inhaled substances that penetrate the mucus layer [106]. Proteolytically active allergens, such as HDM, can disrupt junctional proteins and therefore breach barrier function [27]. In addition, also PM can increase airway epithelial permeability, as demonstrated by a down-regulation or disruption of the adhesion molecule E-cadherin or tight junction proteins, occludin and ZO-1 [107-109]. Moreover, inflammatory mediators and cytokines, such as IL-13, can indirectly disrupt the epithelial barrier integrity [106, 110]. As a consequence, inhaled particles, allergens and viruses can easily penetrate beyond the epithelial surface, facilitating the induction and sustention of innate and adaptive immune responses [106]. Loss of E-cadherin for instance can facilitate allergic sensitization by modulating the DC biology [111]. Furthermore, epithelial barrier disruption can lead to intracellular signaling, as a siRNA knockdown of E-cadherin led to the production of epithelial-derived T_H2 promoting cytokines [112]. In asthmatic airways, evidence of epithelial disruption and fragility has already been demonstrated [102, 104]. In biopsies of human asthmatic patients, a loss of E-cadherin and ZO-1 could be demonstrated compared to non-asthmatic subjects [113].

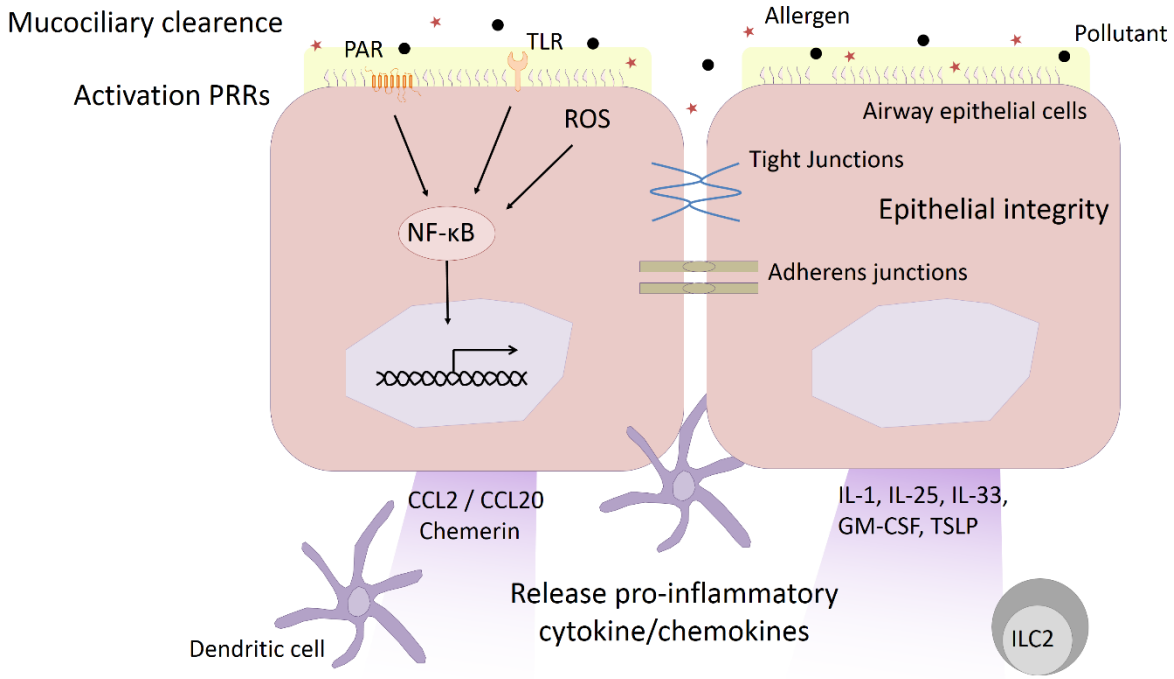


Figure 7: Defensive mechanisms of the airway epithelium against foreign particles. The airway epithelium exerts a mucociliary clearance and functions as a physical barrier through a tight regulation of apical junction complexes (tight and adherens junctions). Moreover, activation of different pattern recognition receptors, expressed on the epithelial cell surface, will lead to the release of multiple pro-inflammatory chemokines and cytokines, contributing to innate and adaptive immune responses. ILC2: Innate lymphoid cell type 2; PAR: Protease-activated receptor; TLR: Toll-like receptor.

Initially, the airway epithelium was considered as a passive barrier, preventing access of harmful agents to the underlying tissue. Nowadays, it is widely accepted that the epithelial layer also has an active role in respiratory defence mechanisms, regulating both innate and adaptive immune responses. Airway epithelial cells express an array of **pattern recognition receptors (PRR)** (i.e. TLR, protease-activated receptor (PAR), NOD-like receptors and C-type lectin receptors) that can detect and respond to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) released upon cellular damage, death and stress [102]. TLR4 triggering on these structural cells for instance was crucial to induce asthma after HDM exposure [114]. Moreover, also ambient PM was found to trigger TLR, including TLR2 and TLR4, to induce airway inflammation [115, 116]. Triggering these PRRs and induction of ROS will induce the activation of nuclear factor κB (NF- κB), leading to the **release of several pro-inflammatory chemokines and cytokines** that can attract and activate immune cells [117, 118]. Epithelial-derived CCL2 and CCL20 for example can be secreted following HDM or DEP exposure to attract monocytes and immature DCs towards the lung [95, 114, 119]. Moreover, the recruitment of DCs can also be mediated by the chemoattractant chemerin that is released from airway epithelial cells upon environmental exposure (see chapter 3.2) [120, 121]. In response to HDM or DEPs the

epithelium can also release several pro-inflammatory cytokines, IL-1, IL-25, IL-33, TSLP and GM-CSF, which can regulate the induction of type 2 immune responses [114, 122, 123] (see addendum, review). These cytokines share the capacity to activate DCs which can promote T_H2 responses in the lung, contributing to the pathophysiology of asthma. Furthermore, recent advances in the field demonstrated that type 2 mediated immunity can also be promoted by the activation of innate immune cells, such as ILC2s (see chapter 4) [102, 117]. The central role of these epithelial-derived cytokines is further supported by the fact that GWAS have identified that polymorphisms in genes encoding for TSLP, IL-33 and its receptor IL1RL1 are associated with asthma risk [16, 17]. Indeed, increased TSLP and IL-33 expression in the airways of asthmatics was already demonstrated, which positively correlated with disease severity [124-126]. The role of IL-33 and its signaling pathway in the lung will be described more in detail below (see chapter 3.3).

3.2 Chemerin

Chemerin, also known as tazarotene-induced gene 2 protein (TIG2), is synthesized as a 163 amino acid precursor, preprochemerin. During secretion, the N-terminal signal peptide is cleaved by a yet unknown protease to form prochemerin with low biological activity [127]. From this precursor, different chemerin fragments can be formed due to various serine proteases, including neutrophil elastase, cathepsin G and mast cell chymase/tryptase [128, 129]. Cleavage at different sites in the C-terminal domain can result into different chemerin peptide isoforms with pro-, anti-inflammation actions or even inactivity [130]. Although chemerin is often described as an adipokine, since one of the predominant sources is the adipose tissue, chemerin was also found in several other tissues, including the lung. The presence of prochemerin could be clearly demonstrated in airway epithelial cells [120, 131] (Figure 8).

Chemerin can bind to three G-protein coupled receptors: chemokine-like receptor 1 (CMKLR1 or **ChemR23**), G protein coupled receptor 1 (GPR1) and chemokine CC motif receptor-like 2 (CCRL2) [130]. Among these receptors, CCRL2 does not promote any cell signaling or receptor internalization upon chemerin binding. It was therefore suggested that CCRL2 increases the local protein concentrations and present chemerin to ChemR23 expressing cells [132]. In contrast, both ChemR23 and GPR1 can induce chemerin-associated signaling, but only ChemR23 will promote cell chemotaxis as GPR1 is not expressed on leukocytes. ChemR23 is expressed on different immune cells, such as immature DCs, monocytes, macrophages and NK cells, which will be attracted to the site of inflammation upon chemerin release, influencing innate and adaptive immunity [130]. Of note, the expression of ChemR23 was also found on endothelial cells [133].

Interestingly, besides chemerin, a second ligand of ChemR23 was identified, resolvin E1 (RvE1), an anti-inflammatory lipid mediator that can promote resolution of inflammation by reducing leukocyte infiltration [134].

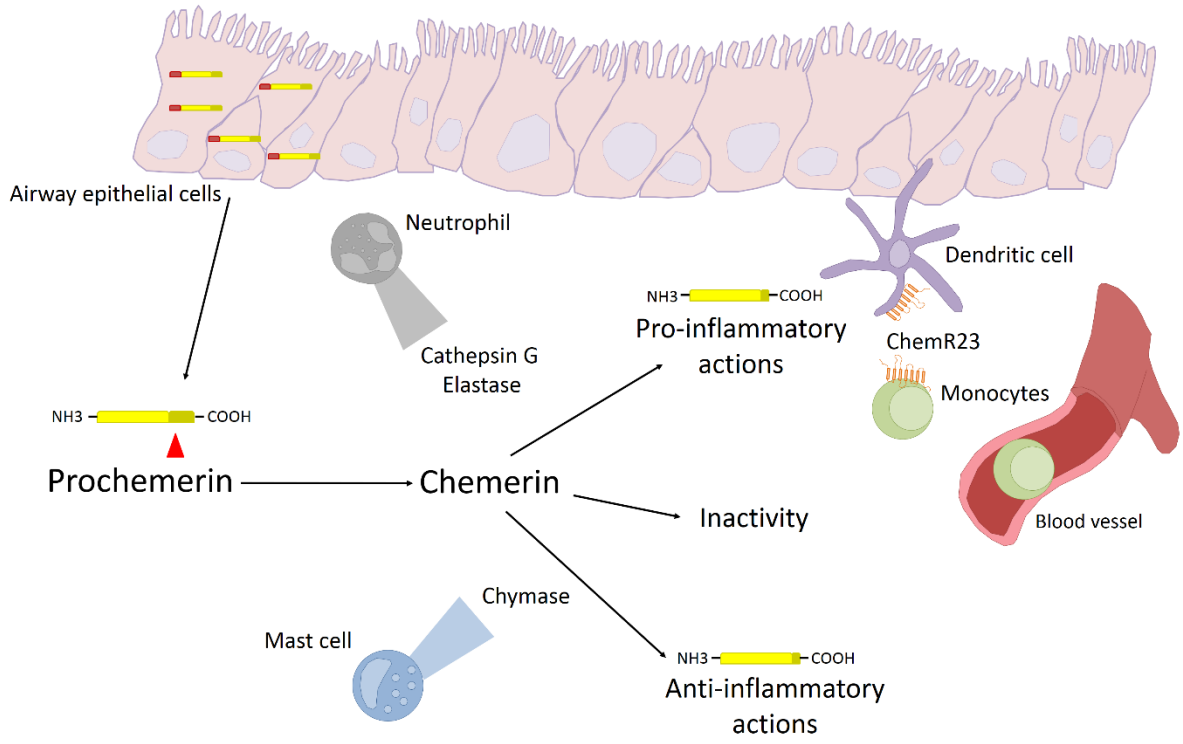


Figure 8: Chemerin/ChemR23 axis in the lung. Preprochemerin, stored in the airway epithelial cells, is released as a poor bioactive precursor, prochemerin. Proteolytic cleavage of the C-terminus by extracellular proteases derived from neutrophils, macrophages and mast cells convert prochemerin into several chemerin variants. Bioactive chemerin is then able to attract and activate ChemR23 expressing cells, such as monocytes, macrophages, NK cells, conventional and plasmacytoid DC. ▬ N-terminal signal peptide. ▲ Cleavage C-terminal domain.

3.2.1 Role of Chemerin/ChemR23 signaling in the lung

The chemerin/ChemR23 axis has gained interest for its multiple roles related to the onset and resolution of inflammation, metabolism and cancerogenesis in multiple organs, including the lung. Depending on the stimulus and which proteases are available in the microenvironment, chemerin exerts pro- or anti-inflammatory actions [130]. As different chemerin variants can be formed, each with their own pharmacological properties, a complex regulatory interaction and therefore different biological functions can be presumed [127].

Previously, our lab demonstrated that cigarette smoke (CS) exposure increased the chemerin levels in the bronchoalveolar lavage fluid (BALF). Moreover, the CS-induced airway inflammation was almost completely abrogated in the BALF and lung of ChemR23 knockout (KO) mice, indicating a **pro-inflammatory role** for chemerin upon CS exposure [120]. Accordingly, a positive correlation between plasma chemerin levels and COPD was recently demonstrated, as elevated chemerin levels could be measured in the serum of COPD patients compared to the control group [135].

In contrast, when ChemR23 KO mice were exposed to LPS, increased inflammatory responses were observed, indicative for **anti-inflammatory properties** of chemerin [131]. Furthermore, ChemR23 KO mice were also more susceptible to viral pneumonia, as a delayed viral clearance, higher mortality and enhanced inflammatory responses were present in the absence of chemerin signaling [136]. In addition, administration of exogenous chemerin attenuated the ovalbumin (OVA)-induced allergic airway inflammation and AHR. These protective properties of chemerin were found to be mediated by a negative feedback mechanism on epithelial CCL2 production, which led to a reduced recruitment of inflammatory DCs to the airways [121].

3.3 Interleukin-33

Interleukin (IL)-33 is identified as a member of the IL-1 family which is composed of 11 cytokines, including IL-1α, IL-1β and IL-18 [137]. Under basal conditions, the IL-33 protein (approximately 31kDa) is constitutively stored in the cell nucleus where it will bind to chromatin, controlling gene expression and maintaining the cells resting state [138]. Its expression is widespread over multiple organs and abundantly found in various cell types, predominantly structural cells such as epithelial barrier tissues and endothelial cells. Although the IL-33 expression is generally not observed in haematopoietic cells, it can be induced under inflammatory conditions, but still at lower levels than in structural cells [139]. Concerning the presence of IL-33 in the lung, important interspecies differences should however be taken into account, as human IL-33 is mainly observed in bronchial epithelial cells and mouse IL-33 is expressed by alveolar type II pneumocytes [139, 140].

In contrast to most cytokines, IL-33 does not contain a signal sequence and will therefore not leave the cell via the conventional endoplasmatic reticulum-Golgi secretory pathway. Instead, IL-33 has been termed an alarmin that is passively released during cellular stress or injury. Recent evidence however suggests that IL-33 can also be actively released, without inducing cell death [139, 140]. It was for instance demonstrated that ATP-dependent IL-33 release can occur in the absence of

cellular necrosis [141]. When released, full length IL-33 is already biologically active, meaning that processing by caspase-1 does not have to occur, in contrast to other family members, such as IL-1β and IL-18 [138]. Of note, it was even demonstrated that IL-33 becomes inactivated after caspase-1 processing [142]. Moreover, also under apoptotic conditions, a programmed cell death where an inflammatory immune response should be avoided, nuclear IL-33 is cleaved and inactivated by caspase-3 and caspase-7 [143].

During inflammation however, the expression of IL-33 can be further increased. Inflammatory proteases derived from neutrophils and mast cells for instance can process full length IL-33 into multiple shorter mature forms (18-21kDa) which possess a 10- to 30-fold greater biological activity than its full length form [144, 145]. Once released, IL-33 can bind with the IL-33 receptor (also known as IL1RL1) that consists of the specific type I transmembrane protein ST2 and interleukin-1-receptor accessory protein (IL-1RAcP). This IL-33R complex is highly expressed by a variety of immune cells, including T cells, ILC2, mast cells, eosinophils, DCs, macrophages, basophils, B cells and NK cells, as well as structural cells like epithelial and endothelial cells [140, 146]. Upon IL-33 binding, signal transduction in the target cell is mediated by the recruitment of MyD88, IRAK and TRAF proteins. These proteins will induce the activation of different MAPK and NK-κB, leading to induction of pro-inflammatory immune responses (Figure 9) [137, 138, 147]. Interestingly, several mechanisms have been developed to limit extracellular IL-33 activity. First, a soluble form of ST2 (sST2) was described that functions as a decoy receptor, neutralizing free IL-33 and therefore preventing IL-33/ST2 signaling [139]. Secondly, shortly after release, IL-33 can be inactivated in the extracellular environment by cysteine oxidation and formation of disulphide bridges, limiting the range and duration of IL-33/ST2-dependent responses [148].

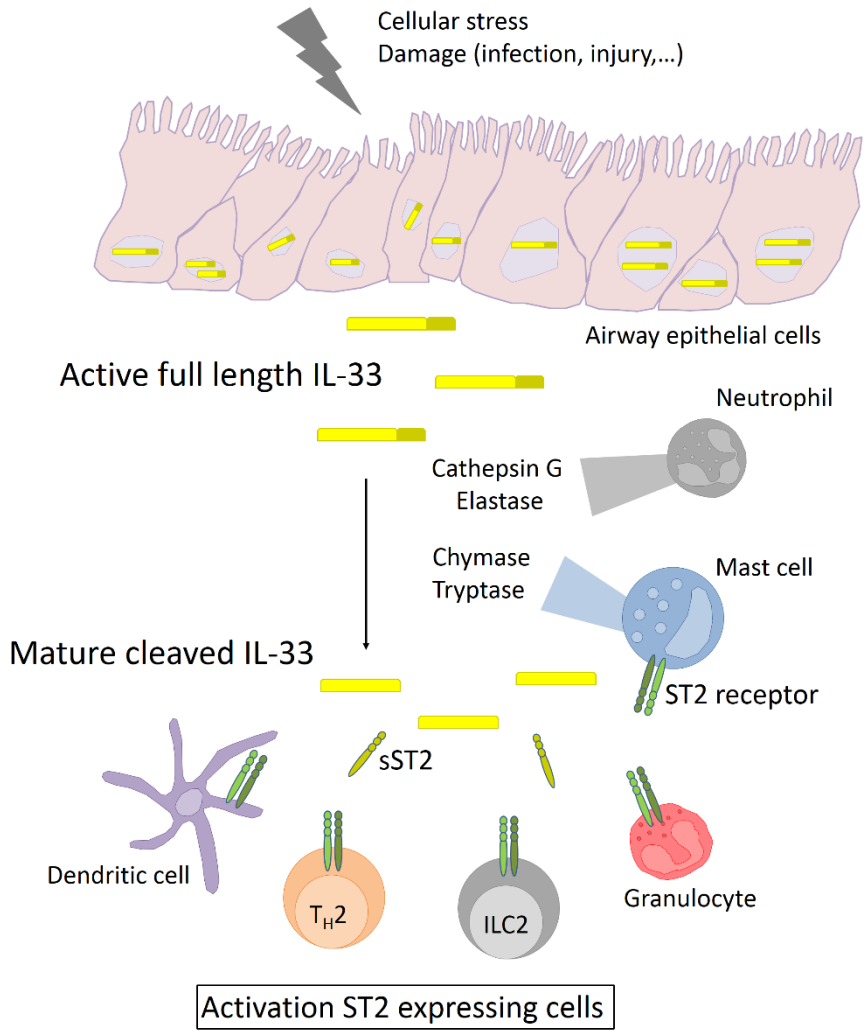


Figure 9: Activation of the IL-33/ST2 axis in the lung. IL-33 is constitutively stored in the nucleus of lung epithelial cells where it controls gene expression and maintains the cells resting state. During mechanical stress and injury, nuclear IL-33 is passively released as a biological active alarmin. Cleavage by several proteases derived from neutrophils, macrophages or mast cells can result into multiple IL-33 isoforms that possess a greater biological activity then its full length form. Once IL-33 is released, it can activate different cells, including mast cells, DCs, macrophages, ILC2, T and B cells, which express the ST2 receptor The activation of the ST2 expressing cells can however be prevented by the presence of soluble ST2 (sST2), which acts as a decoy receptors and neutralizes free IL-33

3.3.1 Role of IL-33/ST2 signaling in the lung

In asthmatics, increased IL-33 expression has been observed in the serum, induced-sputum, BALF and bronchial epithelial cells, which positively correlates with disease severity [125, 149-151]. Moreover, common sequence variants in the *IL-33* and *ST2* gene are associated with **asthma** susceptibility [16, 17, 152, 153]. Recently, also a rare loss-of-function mutation in the *IL-33* gene was discovered which correlated with lower eosinophil counts and protection against asthma [154]. Furthermore, an IL-33 splice variant without exon 3 and 4, resulting in cytosolic localization and facilitated IL-33 secretion, is associated with type 2 airway inflammation in patients with asthma [155].

Most murine studies have identified a critical role for IL-33/ST2 signaling during allergic airway inflammation. Administration of exogenous IL-33 induces goblet cell metaplasia and AHR in mice through type 2 cytokine production [156]. Even in the absence of an adaptive immune system, intranasal administration of IL-33 induced AHR with goblet cell metaplasia and eosinophil infiltration [157, 158]. Moreover, administration of allergens, like HDM, resulted in elevated IL-33 levels shortly after exposure [159-161]. Functional studies using IL-33 KO, ST2 KO mice or recombinant soluble ST2 (r-sST2) demonstrated an attenuation of the HDM-induced allergic airway inflammation and AHR, implicating a critical role for the IL-33/ST2 signaling pathway during allergen-induced airway inflammation [159, 160, 162, 163]. These data indicate that targeting IL-33 could be a new promising therapeutic approach for asthma. Subcutaneous vaccination against IL-33 prior to HDM exposure for instance inhibited the HDM-induced airway inflammation and AHR in mice [164]. Interestingly, it was recently demonstrated that neutralizing IL-33 had the strongest preventive effect on asthma pathology in the mice neonatal period [161]. However, some reports demonstrate an IL-33-independent allergic lung inflammation [165-167]. In the absence of IL-33 for example, Li and his colleagues observed that the overall severity of the HDM-induced allergic airway inflammation was not affected, with the exception of an impaired type 2 cytokine production by ILC2 [165]. At the moment, human studies with regard to anti-IL-33 are ongoing [37, 168]. A recent abstract stated that a human phase I study conducted with anti-IL-33 antibody (ANB020) in healthy volunteers showed a good tolerability and safety profile, supporting the advancement to a phase II clinical trial in severe eosinophilic asthmatics [169].

Mechanistically, IL-33 signaling can contribute to the pathogenesis of asthma by regulating the activity of different immune cells of the innate and adaptive immune system (Figure 10) [140]. Lung ILC2 for instance are a primary target, as they rapidly proliferate and produce typical type 2 cytokines in response to IL-33 [158]. These IL-33-activated ILC2s can be involved in the proliferation and differentiation of $CD4^+$ T cells (see chapter 4) [170]. Moreover, also IL-33-activated DCs can induce the proliferation and differentiation of naïve $CD4^+$ T cells into T_H2 cells [159, 171, 172]. Of interest, IL-33 can also directly influence ST2-expressing T_H2 cells, leading to IL-5 and IL-13 production [173, 174]. In addition, also other immune cells that regulate asthma pathology, including macrophages, eosinophils, basophils and mast cells, can be influenced by IL-33 production [140, 147, 175]. For example, IL-33 contributes to the polarization towards alternatively activated M2 macrophages, which facilitate type 2 immune responses such as airway inflammation [149, 176]. IL-33 also potently enhances eosinophil survival and activation, leading the production and degranulation of several inflammatory mediators, responsible for airway inflammation [177, 178]. Moreover, IL-33 can also act on basophils and mast cells, regulating their adhesion, migration, activation and cytokine production [179-183]. Interestingly, IL-33 can

function in an autocrine fashion on structural cells which also express ST2, leading to enhanced IL-8 production to sustain airway inflammation [184].

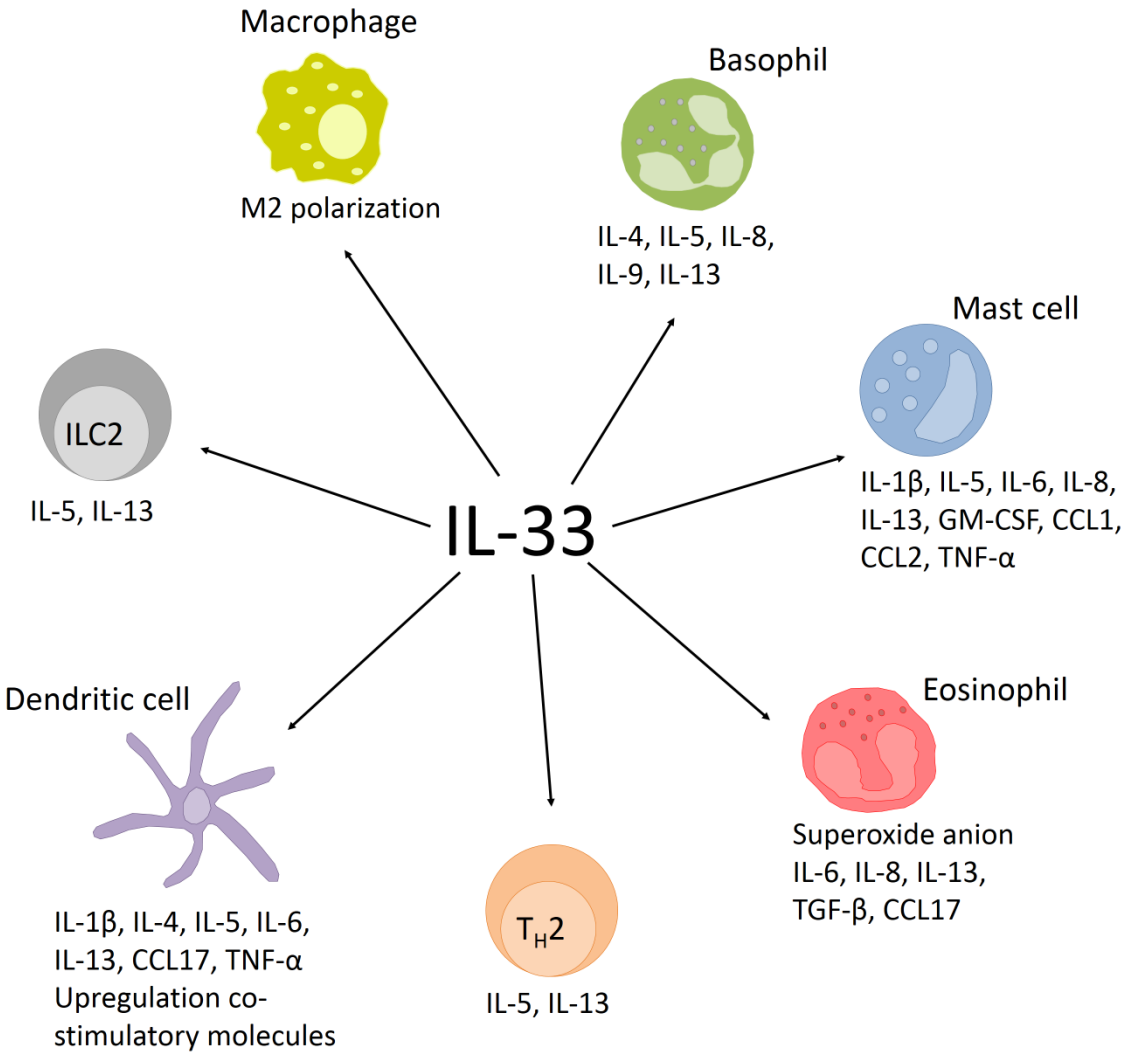


Figure 10: IL-33 regulation of different immune cells involved in the pathogenesis of asthma. In response to IL-33, different immune cells of the innate and adaptive immune system will release an array of cytokines and chemokines, contributing to airway inflammation.

Besides exposure to allergens, also exposure to pollutants, such as DEPs, significantly elevated IL-33 levels, which was associated with functional DC maturation *in vitro* [123]. Moreover, increased IL-33 production was observed when epithelial cells from severe asthmatics were treated with PM [185]. In addition, a critical role for IL-33 was identified during a PM-induced exacerbation in a murine model of chronic allergic airway inflammation [186].

Recently, both human and mouse studies suggested that IL-33 also has an important role in the pathogenesis of **COPD**. IL-33 expression was more abundant in peripheral blood and lung tissue from COPD versus non-COPD subjects, and correlated with disease severity [187-189]. Accordingly, IL-33 and ST2 levels were increased in CS-exposed mice, which was associated with a neutrophilic inflammation and release of pro-inflammatory cytokines [190]. Furthermore, IL-33 was found to be an essential trigger of viral-induced COPD exacerbations by facilitating type I pro-inflammatory responses via an altered ST2 expression pattern in the lungs of CS-exposed mice [189]. Although COPD is typically considered as a neutrophilic-driven airway inflammation, also increased eosinophil numbers have been described in patients with COPD [191]. Indeed, it was recently demonstrated that plasma IL-33 levels in patients with stable COPD positively correlated with the amount of eosinophils [192]. Moreover, the IL-33/ST2 signaling pathway was activated in COPD patients, resulting in a higher secretion of type 2 mediated cytokines [193].

CHAPTER 4: INNATE LYMPHOID CELLS IN CHRONIC AIRWAY DISEASES

4.1 Introduction

Innate lymphoid cells (ILCs) are important early regulators of tissue homeostasis, immunity and inflammation. Although they are morphologically similar to their counterparts of the adaptive immune system, i.e. T and B cells, ILCs lack specific rearranged antigen receptors and are therefore not controlled in an antigen-specific manner. ILCs reside mainly at barrier surfaces, in peripheral tissues such as the lung, intestines and skin. In response to cytokines and inflammatory mediators that are released by epithelial, stromal and myeloid cells, they can shape several immune responses [194, 195]. Importantly, a low number of ILCs have also been described in secondary lymphoid organs, the key site for initiating adaptive immune responses. [196]. In both lymphoid and non-lymphoid tissue, ILCs are tissue-resident cells that are locally renewed and expanded in response to environmental triggers [197].

Analogous with T cells, ILCs are a heterogeneous population which have been classified into three subsets, group 1 (ILC1), group 2 (ILC2) and group 3 (ILC3) ILCs, based on their phenotype, function and transcriptional regulation (Figure 11) [198, 199]. Importantly, ILCs display a high plasticity, meaning that one subset can transdifferentiate into another, allowing them to quickly adapt to environmental changes [194, 200]. Similar to B and T cells, all ILCs arise from a common lymphoid progenitor (CLP). CLP will then develop into common helper innate lymphoid precursors (CHILP), which are characterized by expression of the transcription factor Id2, leading to the development of all non-cytotoxic ILC populations. ILCs lack common lineage markers and depend on IL-7Rα and the IL-2 receptor common gamma chain (γ_c chain) for their development and maintenance [201-203].

Although ILCs are a relatively small population of cells compared with their adaptive counterparts, they appear to have an important role by initiating, regulating and resolving inflammation [204]. ILCs are implicated in pathogen control and tissue repair in the early phases of infection, whereas dysregulation of ILCs can lead to the development of inflammatory diseases. As ILCs can promote disease pathogenesis, modulating their function could have therapeutic potential in several pathological diseases [194, 202]. For the purpose of this thesis, we will shortly discuss the different ILC subsets and subsequently elaborate on their function in lung tissue, specifically focusing on the context of asthma and COPD.

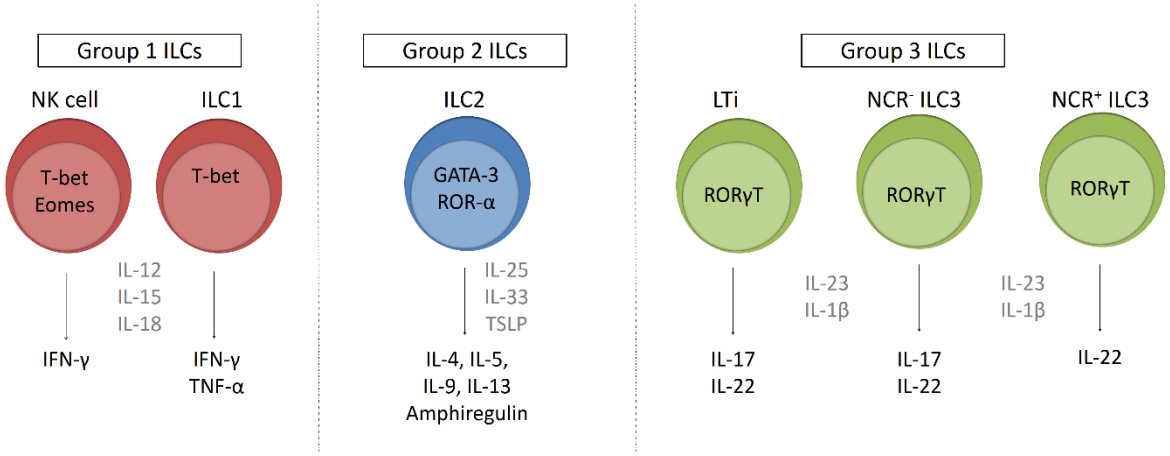


Figure 11: Classification of innate lymphoid cells. Three subgroups of ILC have been defined based on the expression of key transcription factors and cytokines. Group 1 ILC comprise both non-cytotoxic ILC1s and natural killer (NK) cells that express T-bet and produce IFN-γ. ILC2s are defined by the expression of GATA-3 and production of typical type 2 cytokines, such as IL-5 and IL-13. ILC3s are a heterogeneous population including lymphoid tissue inducers (LTi) cells as well as cells that are positive or negative for the natural cytotoxicity receptor (NCR). ILC3s are defined by the expression of RORγT and can produce IL-17 and IL-22.

4.1.1 ILC1

Group 1 ILCs, compromising both non-cytotoxic ILCs and natural killer (NK) cells, are defined as cells that are dependent on the transcription factor T-bet for their development and that produce IFN-γ in response to IL-12, IL-15 and IL-18 (Figure 11) [198, 199]. Although the development of NK cells does not strictly depend on T-bet but also on the transcription factor Eomes, they are still categorized as group 1 ILCs as they can rapidly produce large amounts of IFN-γ [205]. While NK cells recirculate between the blood and the tissue, ILC1s are presumed to be tissue resident cells [206]. Despite some clear developmental differences between NK cells and non-cytotoxic ILC1s, they are difficult to distinguish in most tissues as they share many features and their identification markers can be modulated under conditions of infection and inflammation [203, 205]. Moreover, ILC1s are a considerably heterogeneous population, including a mix of ex-ILC2s and ex-ILC3s [207], providing additional challenges to distinguish between the different ILC1 subsets. Besides the role of ILC1s in the defence against intracellular pathogens [204], they are also considered as possible disease modulators since increased numbers of ILC1s are observed in several inflammatory diseases, including COPD, Crohn's disease, rheumathoid arthritis, spondyloarthritis, lupus and systemic sclerosis [202].

4.1.2 ILC2

ILC2s, initially termed natural helper cells or nuocytes, depend on the transcription factor GATA-3 and ROR-α for their development and are a potent source of typical type 2 cytokines especially IL-5 and IL-13. They rapidly expand and become activated in response to several epithelial-derived alarmins, including IL-25, IL-33 and TSLP, orchestrating early type 2 immune responses at mucosal surfaces (Figure 11) [198, 199]. To date, two ILC2 developmental pathways are being proposed, giving rise on the one hand to homeostatic ILC2s (natural ILC2, nILC2), sensitive to IL-33, and on the other hand IL-25 responsive-inflammatory ILC2s (iILC2) [208]. Under inflammatory conditions, ILC2s can also become activated by prostaglandin D2 (PGD2) and cysteinyl leukotrienes (CysLTs) [201, 209-211]. Interestingly, ILC2s can also produce large amount of IL-9 that in an autocrine fashion potentiates type 2 cytokine production and accounts for ILC2s survival by inhibiting apoptosis [201, 212]. Depending on the microenvironment and inflammatory stimulus, ILC2s can exert both protective and pathogenic effects. ILC2s can exert host-protective functions against helminth infections and promote resolution of inflammation by restoring epithelial integrity and promoting tissue repair [201, 202, 204, 213, 214]. In contrast, dysregulation of the ILC2 responses can contribute to several inflammatory diseases, such as asthma, chronic rhinosinusitis and atopic dermatitis [202, 215]. As a consequence, ILC2s or pathways related to ILC2 activation have been suggested to be potential therapeutic targets in allergic diseases (see chapter 4.2.1).

4.1.3 ILC3

ILC3s are a heterogeneous population composed of at least three different subtypes, including natural cytotoxicity receptor (NCR)-positive ILC3s and NCR^- ILC3s which also include lymphoid tissue inducer cells (LTi). ILC3s express the transcription factor RORγT and can produce IL-17A, IL-17F and IL-22 in response to IL-23 and IL-1β (Figure 11) [198, 199]. LTi cells are a special subset of ILC3s which were initially described as initiators of lymphoid organogenesis. Indeed, during fetal development, ILC3s are required for the generation and maturation of secondary lymphoid structures, such as lymph nodes and Peyer's patches. Similarly, ILC3s are important for lymphoid tissue formation during adult life, leading to cryptopatches (an aggregate of lymphoid cells located in the intestinal lamina propria) and formation of tertiary lymphoid structures, also known as isolated lymphoid follicles [216-218]. Considering that LTi are clustered within lymphoid tissues, they can orchestrate adaptive immune responses, supporting B cell class switching, antibody production and $CD4^+$ T cell modulation [218, 219]. Interestingly, ILC3s are

also an important source of GM-CSF, promoting tolerogenic responses to commensal bacteria [220]. ILC3s are key regulators of mucosal barrier tissue homeostasis, inducing antimicrobial resistance and maintaining the epithelial barrier function by regulating tissue repair [204, 218, 221]. Alterations of ILC3s are described in a diversity of inflammatory diseases, including inflammatory bowel diseases, psoriasis and rheumatoid arthritis [202].

4.2 ILCs in the lung

Although ILCs are a relatively rare population, all three ILC populations are present in human lung tissue under homeostatic conditions [194]. In pulmonary diseases however, ILC responses can substantially alter, contributing to the pathological processes in the lung [222]. In the next sections, we will elaborate on the role of ILCs in asthma and COPD.

4.2.1 ILCs in asthma

4.2.1.1 Activation of ILC2s

In the pathogenesis of asthma, especially the involvement of ILC2s has been explored, as several pro-inflammatory cytokines (i.e. IL-25, IL-33 and TSLP) and lipid mediators (CysLTs and PGD2), which can activate ILC2s, were elevated in asthmatic airways [125, 126, 223-226]. Moreover, polymorphisms in genes encoding TSLP, IL-33, IL-33 receptor, IL-13 and RORα, which are implicated in ILC2 development or function, have been associated with asthma susceptibility [16, 17]. Indeed, in patients with asthma a higher number of total and type 2 cytokine producing ILC2s were detected in the sputum, BALF and blood [227-233]. Interestingly, an association between activated ILC2s and asthma control status was proposed, as the number of IL-13-producing ILC2s rapidly decreased when the patient's symptoms were under control, suggesting that ILC2s could serve as a predictor of asthma control status [231]. Moreover, recent evidence suggests that ILC2s could also have an important role in paediatric asthma, as ILC2s could be demonstrated in the BALF, induced sputum, and peripheral blood from children with severe therapy-resistant asthma [232].

In murine studies, it is demonstrated that in response to various allergens, ILC2s accumulate in the lung and are major producers of IL-5 and IL-13 [234]. The expansion of IL-13-producing ILC2s is more profound in response to IL-33, compared to IL-25 [235]. Moreover, epithelial-derived TGF-β, which can be activated by IL-33, appears to be essential for ILC2 activation and

accumulation in the early allergic responses [236]. Interestingly, an optimal induction of ILC2 activation and the subsequent inflammatory responses is proposed to depend a coordinated response of all epithelial cytokines [237]. Furthermore, in newborn mice, it is demonstrated that ILC2s spontaneously accumulate in the developing lung in an IL-33 dependent manner. Early HDM exposure can then further increase IL-33, leading to the activation of these ILC2s, promoting early life type 2 immunity [161, 238]. Of note, in addition to epithelial-derived cytokines, also mast cell mediators, such as PGD2 and LTD4, can drive ILC2 activation [210, 239]. Moreover, basophils can promote ILC2 proliferation by secreting IL-4 in response to protease allergens [240] (Figure 12).

4.2.1.2 Role of ILC2s in the initiation of allergic airway inflammation

The functional importance of ILC2s in asthma was first suggested by studies in RAG2 KO mice, as even in the absence of an adaptive immune system, ILC2s are able to mediate airway eosinophilia, goblet cell metaplasia and AHR in response to allergen inhalation or recombinant IL-33 [157, 158, 241, 242]. These allergic airway responses are however abrogated in $RAG2^{-/-}IL2rg^{-/-}$ mice that lack all ILC populations, and can be restored after adoptive transfer of lung ILC2s, enabling these mice to respond to allergen exposure [241, 243]. Moreover, type 2 lung inflammation is also abrogated in RORα-deficient mice, which lack specifically the ILC2 population [244, 245].

ILC2s influence multiple cell types by direct cell contact or indirectly through the secretion of soluble cytokines, thereby promoting airway inflammation [246, 247]. Recent data indicate that specifically the crosstalk between ILC2s and T cells is crucial for their maintenance, proliferation and activation (Figure 12) [248]. ILC2s are found to be required in promoting T_H2-cell-mediated allergic lung responses, as mice lacking ILC2 have impaired T_H2 cell immunity [170, 245, 249]. A critical role for DCs is herein suggested, since ILC2-derived IL-13 can promote the migration of DCs towards the MLN, leading to T_H2 differentiation [170]. Moreover, upon allergen re-challenge, this ILC2-DC axis is also required to recruit memory T_H2 cells to the inflamed lung tissue [250]. Importantly, ILC2s and $CD4^+$ T cells can also directly interact with each other via OX40 and MHCII expression on ILC2s, leading to T cell activation [249, 251, 252]. In turn, activated T cells can secrete IL-2, promoting ILC2 proliferation and cytokine production [249, 252]. However, in some murine models ILC2s are dispensable for the induction of adaptive immune responses. In contrast to local antigen exposure, systemic priming with ovalbumin for instance did not require the presence of ILC2s for the induction of T_H2-driven lung inflammation [245]. Moreover, during HDM-driven allergic airway inflammation, T cell activation precedes ILC2 induction [165]. In

addition to the effects on T cell function, ILC2s can also promote the activation of eosinophils, alternatively activated macrophages and B cells [245, 253, 254].

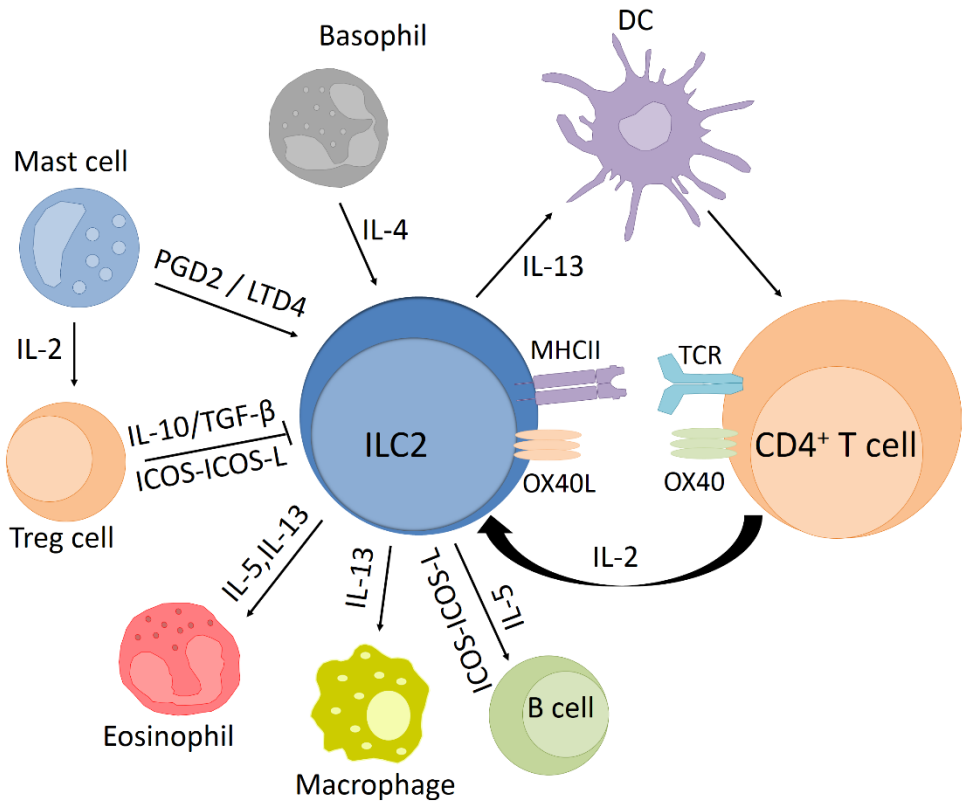


Figure 12: Crosstalk of ILC2s with cells of the innate and adaptive immune system. ILC2s can interact with other effector immune cells by direct cell contact or through the secretion of soluble mediators.

As previously stated, in addition to allergens, also non-allergic factors, such as air pollutants, can initiate and exacerbate the pathogenesis of asthma [60]. To date, ILC2s have been shown to mediate ozone-induced airway inflammation and AHR [255, 256].

4.2.1.3 Role of ILC2s in the persistence of allergic airway inflammation

Besides the role of ILC2s in the initiation of allergic airway inflammation, also the persistence of several asthmatic features, including lung inflammation, AHR and remodelling, can depend on ILC2s. Critical feedback mechanisms between epithelial cells and ILC2s for instance generate mechanisms for sustained IL-33 production, leading to a chronic airway inflammation [151]. Moreover, memory-like properties of lung ILC2s are recently demonstrated, as some ILC2s persist long after the resolution of allergen-mediated airway inflammation and respond more vigorously

upon reactivation compared to naïve ILC2s [257]. In asthmatics, type 2 inflammation can be sustained by ILC2-derived IL-13 which disrupt the integrity of the bronchial epithelium by targeting the tight junctions [258].

4.2.1.3 Suppression of ILC2s

Importantly, ILC2 function can be suppressed by soluble mediators or direct cell contact. Type I interferons (IFN), type II IFNs and IL-27 can counter the proliferation and activation of ILC2s in a STAT1-dependent manner [259]. Furthermore, IFN-γ can suppress the IL-9 production of ILC2s in a T-bet-dependent fashion, inhibiting ILC2s survival [260]. Moreover, suppressive effects have been attributed to regulatory T (Tregs) cells. IL-10 for instance, produced by elevated Treg numbers in response to IL-2 release by IL-33 stimulated mast cells, can inactivate ILC2s and IL-33- or papain-induced airway inflammation [243]. Moreover, the suppressive effects of Tregs on ILC2 activity can also be mediated through ICOS-ICOSL cell contact or TGF-β-signaling [261, 262] (Figure 10). Lastly, some eicosanoids, which are derived from arachidonic-acid, can negatively regulate ILC2 function. Prostaglandin I2 (PGI2) for example exerts predominantly anti-inflammatory effects, leading to an inhibition of ILC2 function. Administration of a PGI2 analog (cicoprost) inhibits IL-33-induced ILC2 proliferation in mice and decreases type 2 cytokine production in humans [263]. Moreover, also lipoxin A4 (LXA4) functions as a negative regulator of ILC2s, inhibiting their type 2 cytokine production [264].

4.2.1.4 ILC2s as therapeutic targets

Considering the growing evidence on the importance of ILC2s in asthma pathogenesis, ILC2s or ILC2-releated pathways are considered as potential therapeutic targets [202]. By targeting lipid mediators and their receptors for instance, ILC2 function can be modulated [194, 202, 215]. Prostaglandin D2 receptor (CRTH2) or leukotriene receptor antagonists (montelukast) can reduce eosinophilic airway inflammation, improve lung function or prevent ILC2 activation respectively [265-267]. Moreover, strategies that negative regulate ILC2 function, such as LXA4, PGI2 and Tregs, could be employed to inhibit ILC2-driven inflammatory responses [263, 264, 268]. As severe asthmatics can have a LXA4 deficiency [269], LXA4 supplementation could be beneficial for these patients. Intriguingly, in severe non-allergic eosinophilic asthma, which is often insensitive to corticosteroid treatment and is associated with exposure to air pollutants, microbes and glycolipids, the hypothesis was raised that especially ILC2s will be of interest, rather

than T_H2 immune cells [270]. Although there is still some controversy, it is indeed suggested that ILC2s are insensitive to steroids, as ILC2s numbers were still increased in the airways of severe asthmatics despite high doses of oral corticosteroids [230]. Drugs that target TSLP or STAT5 have been proposed to overcome the resistance to corticosteroid treatment [271]. Interestingly, in a phase II clinical trial, anti-TSLP was recently demonstrated to be very effective in severe uncontrolled asthmatics, leading to significant fewer asthma exacerbations compared to placebo [50]. Strategies that target TSLP and other epithelial-derived cytokines, i.e. IL-25 and IL-33, need further investigation [194, 202, 215] and combinatorial blockage of all epithelial-cytokines may be necessary to effectively inhibit ILC2-mediated lung inflammation [272]. Lastly, as described earlier (see chapter 1.2.3), blocking downstream cytokines, such as IL-5, has proven to be therapeutically effective in the treatment of severe eosinophilic asthma [45]. Further studies are however required to elucidate the direct effects of these treatments on ILC2s since they can also target other inflammatory cells.

4.2.1.5 Other ILC subsets in asthma

Emerging studies have unravelled a possible association between asthma and the presence of ILC3s. In severe asthmatics, an increased amount of IL-17-expressing ILC3s was found in the BALF compared to mild asthmatics and healthy volunteers [273]. Moreover, IL-17-expressing ILC3s were increased in paediatric severe therapy resistant asthma [274]. In a murine study, IL-22-producing ILC3s were proposed to modulate allergic responses in the lung, limiting airway inflammation and contributing to the maintenance of homeostasis [275]. Whereas for the moment no data are available concerning non-cytotoxic ILC1s in the pathogenesis of asthma, the role of NK cells has been better studied [276]. It was for instance suggested that NK cells could be critical for the development of allergen-induced AHR [277].

4.2.2 ILC in COPD

To date, limited studies are performed to assess the abundance and functions of ILCs in COPD. In GOLD stage IV patients, a lower amount of ILC2s were detected, whereas significantly more ILC1s and ILC3s were present compared to healthy subjects or patients with less-severe COPD (GOLD I and II). It was here suggested that the decrease in ILC2s was due to the transdifferentiation into ILC1s [278]. Moreover, the augmented ILC1 responses correlated with disease severity and susceptibility to exacerbations [279]. In accordance, CS silences murine ILC2 function, which

facilitated an exaggerated type 1 anti-viral response, leading to exacerbating disease [189]. Recently, ILC3s were detected in inflammatory aggregates in lungs of smokers and COPD patients, suggestive for the contribution of ILC3s in the formation of lymphoid aggregates and follicles [280].

CHAPTER 5: TRANSLATIONAL RESEARCH – MATERIALS AND METHODS

5.1 Translational Research

Translational research refers to the process of applying discoveries generated through laboratory research into the development of new drugs and treatments which will be tested in clinical trials (from bench to bedside) (Figure 13). Applying the obtained findings into medical practice is the ultimate goal of translational research.

Figure 13: Translational Research. The translation of laboratory discoveries to the clinic (bench to bedside) and new therapies applying to clinical practice, populations based studies (bedside to community).

In our lab, we have optimized 3 different approaches, which will be discussed in detail in this thesis, to elucidate several research questions. In basic research, **murine models** are a valuable tool to test hypotheses and give more insights into the molecular and cellular mechanisms. Considering that murine models of asthma closely mimic important features of the disease, such as airway inflammation, remodeling and hyperresponsiveness, they are often employed in asthma research. Availability of genetic manipulated mouse strains allows researchers to investigate the involvement of specific genes or molecules *in vivo*. Moreover, mice are relatively easily bred, housed and handled which make them an ideal *in vivo* research model. Although the mouse genome is completely sequenced and is highly homologue with the human genome, there are several limitations that have to be taken into account when working with animal models. Especially the differences in the respiratory anatomy, physiology and immune system could have implications on the outcome, making the translation of murine findings to the human disease difficult [23]. Moreover, ethical concerns are being raised towards animal experimentation. Therefore, in addition to our murine model, an *in vitro* approach with primary **human bronchial epithelial cell (HBEC)** cultures can be employed. These *in vitro* cell cultures allow to investigate specific mechanisms of action concerning the human epithelium. It has to be acknowledged however that observations made in these *in vitro* settings could differ from the *in vivo* situation, since these cells grow in an artificial milieu without cell to cell interactions which differs from

what they encounter in an *in vivo* situation. In a third approach, knowledge that has been obtained in basic research can be validated on ***ex vivo* human lung samples** from patients. These samples can be used to characterize different human cell populations and study gene and protein expression.

In this thesis, we are interested in the aggravating effects of DEPs on allergic airway inflammation. Therefore, we have optimized an *in vivo* murine model where DEPs were given simultaneously with a clinical relevant allergen, house dust mite (HDM) (see 5.2.2). Moreover, to characterize the recently described innate lymphoid cells populations in human lung, we have used *ex vivo* human lung samples from patients with COPD, smokers without airflow limitation and never smokers (see 5.3.1).

5.2 Murine models

5.2.1 Murine model of acute DEPs exposure

To investigate the mechanisms of DEPs on the respiratory system, Provoost S *et al.* established an acute DEPs model in our lab wherein the acute effects of DEPs on the innate immune responses are closely mimicked. DEPs (SRM 2975) were purchased from the National Institute for Standards and Technology (NIST, Gaithersburg, MD) and suspended in saline containing 0,05% tween 80. Ketamine/xylazine anaesthetized mice are intratracheally instilled with saline or 100µg DEPs in a 50µL volume on day 1, 4 and 7. On day 9, the inflammatory mediator release and cell recruitment are assessed. Tween 80 (0.05%) was used as a vehicle in all conditions [94].

5.2.2 Murine model of DEPs-enhanced allergic airway inflammation

To unravel the mechanisms underlying the enhanced effects of DEPs on allergic airway inflammation, a clinical relevant combination model of DEPs with HDM was developed. HDM (Dermatophagoides pteronyssinus) was obtained from Greer laboratories (Lenoir,NC). This DEPs+HDM combination model was developed from hands-on experience with an in-house CS+HDM that had been established earlier [281]. First of all, dose titration experiments were performed since the enhancing capacity of DEPs on allergic airway inflammation can no longer be observed when mice are exposed to too high or low doses of both DEPs and HDM [96, 98]. Ultimately, intranasal application of saline, 25µg DEPs, 1µg HDM dissolved in saline and combined DEPs+HDM is performed in isoflurane anaesthetized mice by using a continuous flow vaporizer on day 1, 8 and 15. Experimental endpoints are performed on day 17. Tween 80 (0.05%) was used as a vehicle in all conditions.

5.2.3 Endpoints and analyses

48 hours after the last exposure, mice are anaesthetized for lung function measurements or sacrificed with a lethal dose of intraperitoneal (IP) pentobarbital. Murine blood, brochoalveolar lavage fluid (BALF), lung tissue and mediastinal lymph nodes (MLN) are collected to assess the inflammatory responses (Figure 14). All *in vivo* manipulations were approved by the local ethical committee for animal experimentation of the Faculty of Medicine and Health Science, Ghent University.

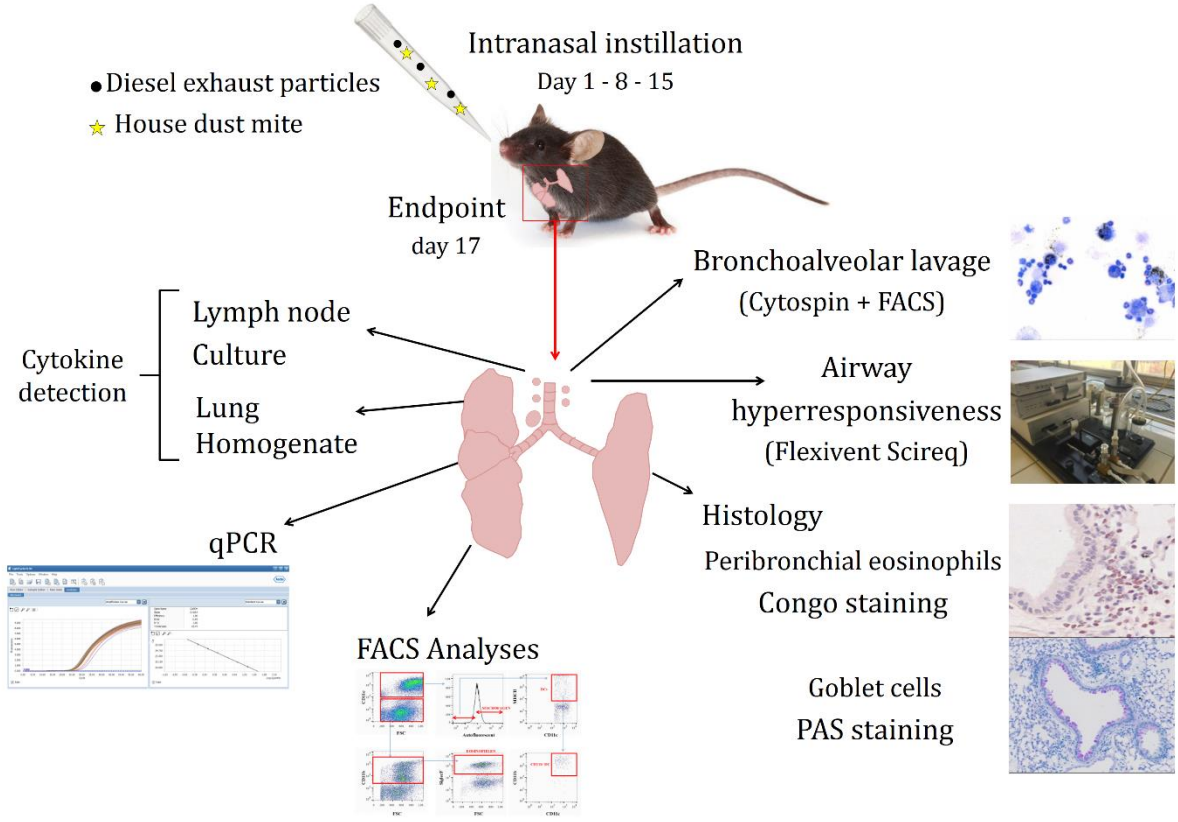


Figure 14: Overview experimental techniques on DEPs+HDM exposed mice. C57BL/6 mice are intranassaly instilled with DEPs+HDM on day 1, 8 and 15. 2 days later endpoints are performed to examine the airway responses. Bronchoalveolar lavage is assessed by cytospin and FACS analyses. The airway hyperresponsiveness is evaluated with the forced oscillation technique. Cytokine responses are analyzed in the lungs and lymph nodes that are restimulated with HDM *in vitro*. The inflammatory responses in the lung are also analyzed with FACS and RNA extraction is performed to perform RT-PCR. Moreover, peribronchial eosinophils and goblet cells are detected by histological analyses.

5.2.3.1 Lung function measurements

Lung function measurements are assessed using the forced oscillation technique (Flexivent system, SCIREQ, Montreal, QC, Canada) (Figure 15). Airway hyperresponsiveness (AHR) is assessed in mice using carbachol, which provokes bronchoconstriction by directly activating the airway smooth muscle cells. In brief, neuromuscular blockade is induced by intravenously (IV) injecting pancuronium bromide (1mg/kg) to anaesthetized and tracheostomized mice. The airway resistance of the whole respiratory system (airways, lungs and chest wall) is measured during a "snapshot perturbation" manoeuvre, while mice are challenged with increasing doses of carbachol (0-640 µg/kg). For each concentration, 12 "snapshot perturbations" are performed and the % increase in lung resistance is calculated relative to the baseline resistance.

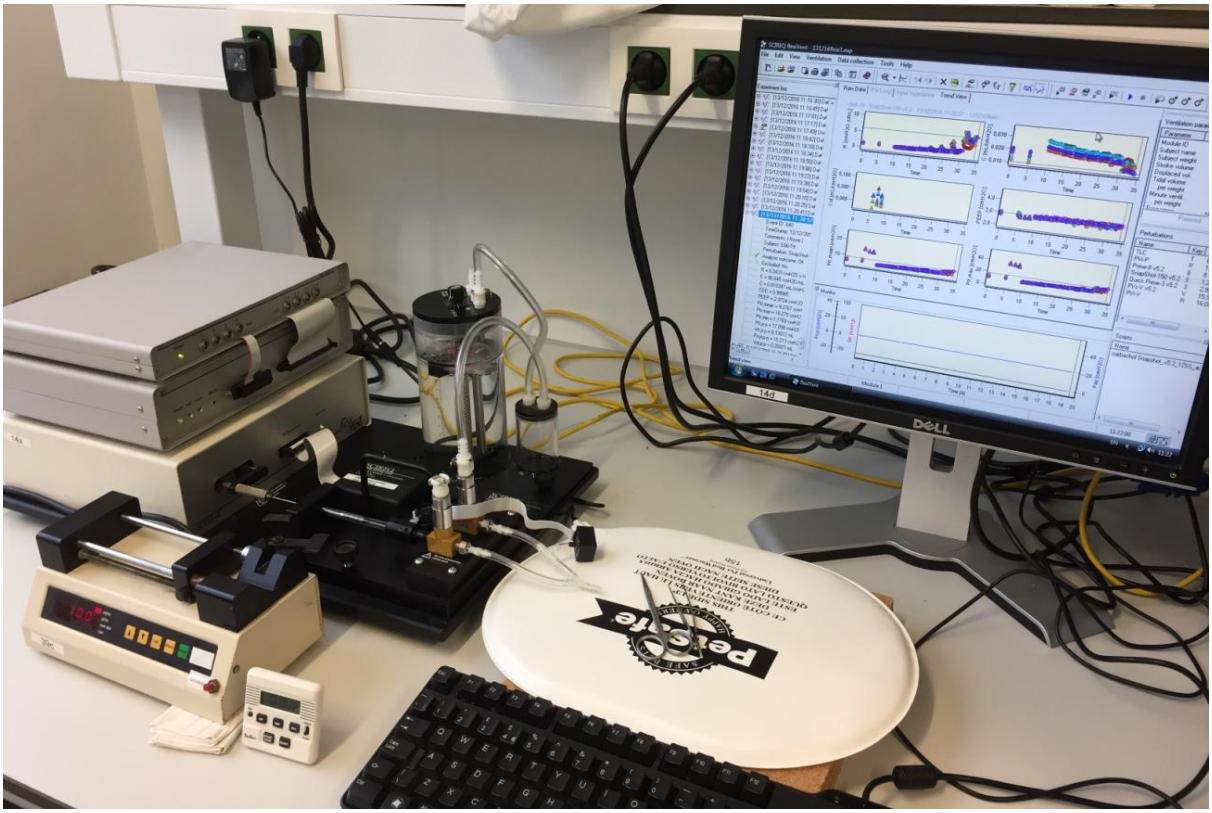


Figure 15: Flexivent system for lung function measurements.

5.2.3.2 Bronchoalveolar lavage fluid (BALF)

Inflammatory cells from the bronchial and alveolar spaces of the lung are withdrawn by performing a bronchoalveolar lavage (BAL). Therefore, a tracheal cannula is inserted and a salt solution is gently instilled and withdrawn. The cell-free supernatants are stored to assess cytokines by ELISA (see 5.2.3.7). The cell pellet is used for flow cytometric analyses (see 5.2.3.3) and cell differentiation on a cytospin using a May-Grünwald Giemsa staining. The use of cytospins allows discrimination of monocytes/macrophages, neutrophils, eosinophils and lymphocytes (Figure 16).

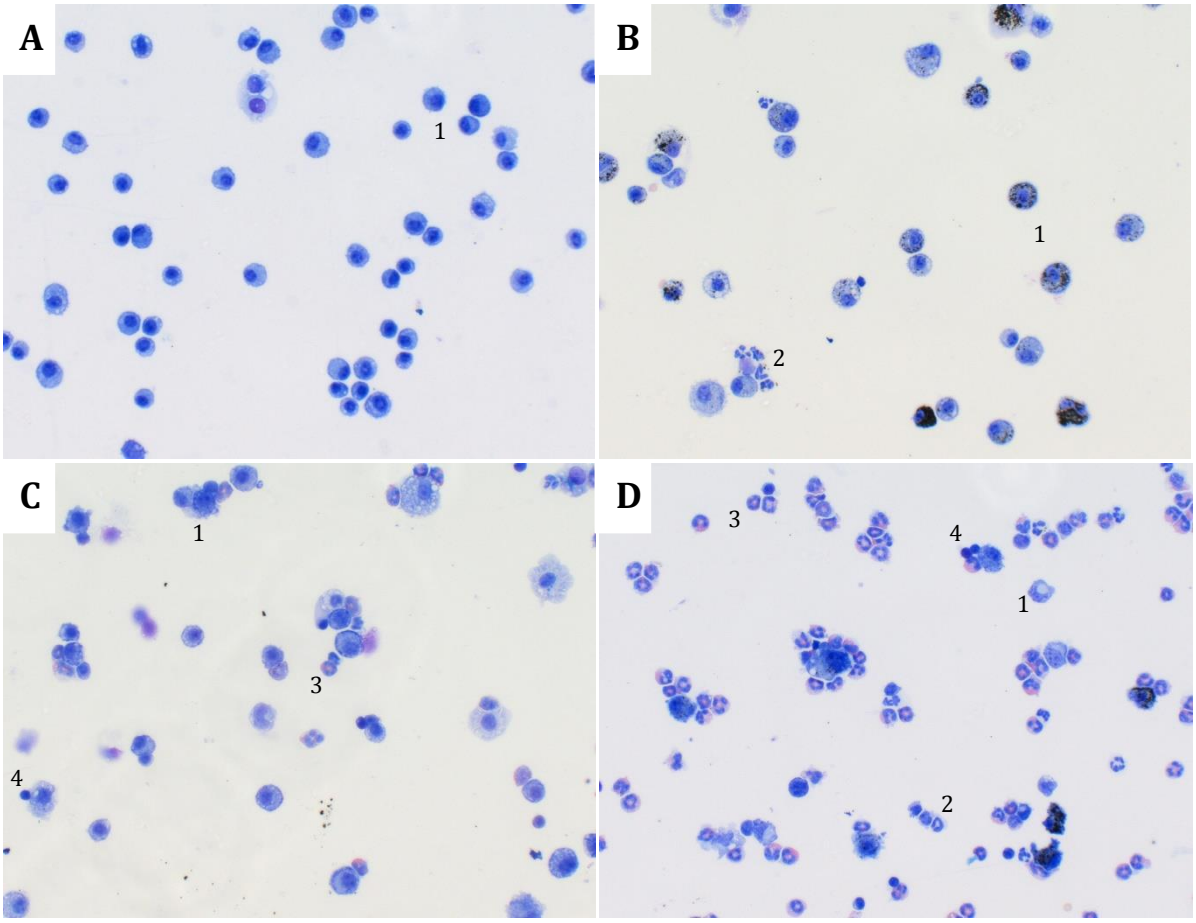


Figure 16: Cytospins of bronchoalveolar lavage cells stained with May-Grünwald Giemsa. Representative photomicrographs of BALF cells of **A)** saline, **B)** DEPs, **C)** HDM or **D)** DEPs+HDM-exposed mice. 1= monocyte/macrophage, 2= neutrophil, 3= eosinophil, 4 = lymphocyte.

5.2.3.3 Flow cytometry

BALF cells and single cell suspensions from the major lobe of the right lung are used for flow cytometry. This technique allows the characterization of multiple cell types based on the combination of specific surface and intracellular markers. Therefore, antibodies conjugated to different fluorescent dyes are used. Excitation of these fluorescent dyes by a laser will transmit characteristic emission spectra which will be measured by the flow cytometer. The cells that are described in this thesis are summarized in figure 17.

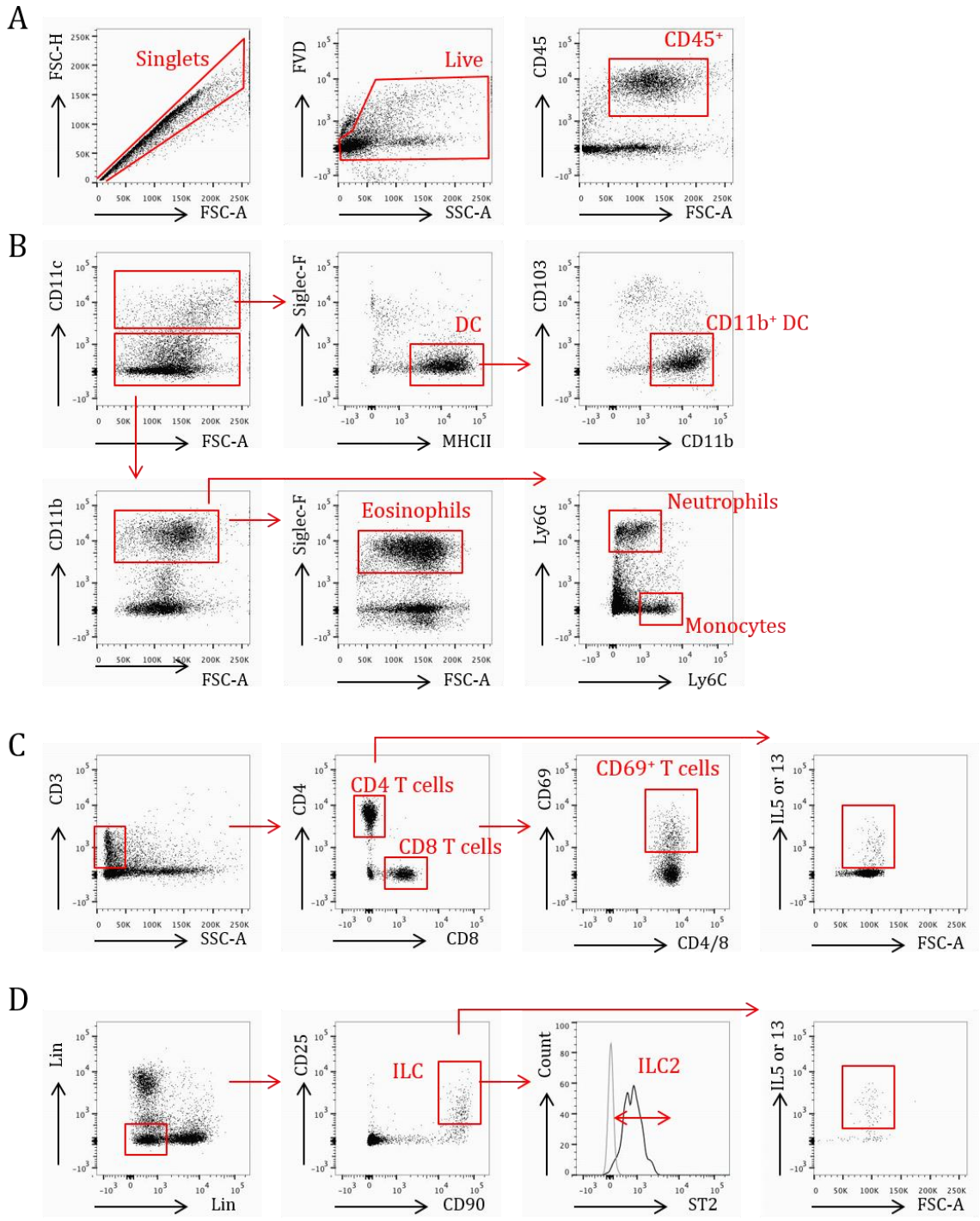


Figure 17: Gating strategy inflammatory cells in mice (lung tissue). Representative dot plots of WT mice that were exposed to combined DEPs+HDM. **A)** Selection of single cells, Fixable Viability Dye (FVD) negative cells and CD45$^+$cells was made for all cell types. **B)** DCs were identified as CD11c$^+$, Siglec-F$^-$, MHCII$^+$ and CD11b$^+$. Eosinophils were characterized as CD11c$^-$; CD11b$^+$ and Siglec-F$^+$. Neutrophils were gated as CD11c$^-$, CD11b$^+$, Ly6G$^+$ and Ly6C$^+$. Monocytes were CD11c$^-$, CD11b$^+$, Ly6G$^-$ and Ly6C$^+$. **C)** T lymphocytes were identified as CD3$^+$ cells. A distinction was made between CD4$^+$ and CD8$^+$ T cells. CD69 was used as an activation marker. Intracellular IL-5 and IL-13 in CD4$^+$ T cells was investigated. **D)** Innate lymphoid cells (ILC) type 2 were characterized as Lin$^-$ (CD3, CD5, TCRβ, NK1.1, CD11c, CD11b and CD45R), CD25$^+$, CD90$^+$ and ST2$^+$ cells. Intracellular IL-5 and IL-13 in ILC was investigated.

5.2.3.4 Mediastinal lymph node (MLN) culture

MLN single cell suspensions are cultured *in vitro* to assess the cytokine expression within the draining lymph nodes. Therefore, 200.000 cells/well are cultured in 96 well plates, either alone or supplemented with 3.75µg/well HDM. After 5 days in a humidified 37°C incubator and 5% CO_2 atmosphere, supernatant is harvested for cytokine measurements with ELISA (see 5.2.3.7).

5.2.3.5 Histological evaluation

To quantify peribronchial eosinophils and goblet cell metaplasia, paraffin sections of the left lung are subjected to a chemical staining, Congo Red and Periodic acid-Schiff (PAS) respectively. Quantitative measurements are performed in a blinded fashion on a Zeiss Axioimager running Axiovision software. Periobronchial eosinophils are determined as the total number per mm^2 bronchial wall area (WAt), whereas PAS-positive cells are expressed per millimeter perimeter of the basement membrane (Pbm) (Figure 18). Airways with a basement membrane < 800 µm or > 2000 µm are excluded. Measurements from at least 10 airways per mouse are performed.

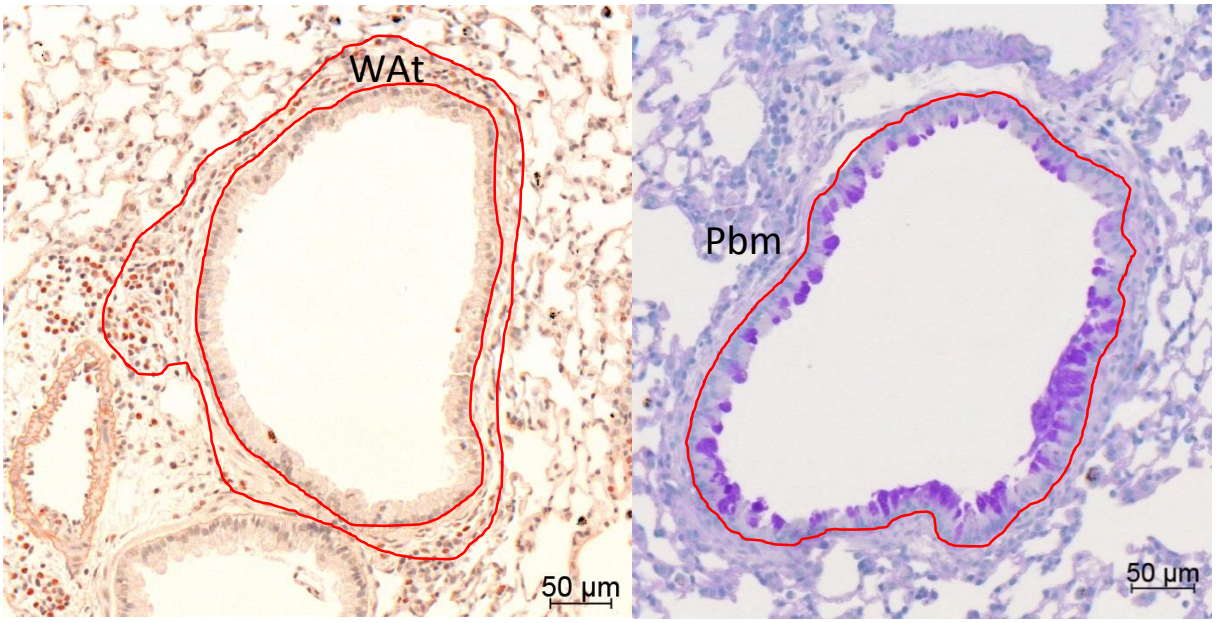


Figure 18: Quantification of peribronchial eosinophils and goblet cell metaplasia. Representative photomicrographs of **A)** congo and **B)** PAS stained lungs of DEPs+HDM-exposed mice. WAt = total bronchial wall area, Pbm = perimeter of the basement membrane.

5.2.3.6 qRT-PCR

Quantitative real time polymerase chain reaction (qRT-PCR) is a powerful tool to quantify gene expression profiles. Briefly, lung mRNA is extracted using the miRNeasy Mini kit (Qiagen) and cDNA is prepared with the Transcriptor Universal cDNA Master kit (Roche) following the manufacturer's instructions. The gene of interest relative to housekeeping genes is analysed using Taqman Gene Expression Assays (Life Technologies). qRT-PCR is performed on a LightCycler 96 detection system (Roche).

5.2.3.7 ELISA

Enzyme-linked Immunosorbent Assay (ELISA) is an effective method which allows the quantification of proteins in serum, lung homogenate, BALF and MLN supernatants. Immunoglobulin levels are determined on serum collected by retro-orbital bleeding. Total IgE is measured using LO-ME-3 coated plates and biotinylated polyclonal rabbit anti-mouse IgE (S. Florquin, Universite´ libre de Bruxelles). For detection of HDM-specific IgG1, plates are coated with 25µg/ml HDM extract. Serum is added, followed by a horseradish peroxidise (HRP)–conjugated polyclonal goat anti-mouse IgG1 antibody (Bethyl Laboratories, Montgomery, Tex). Ig levels are reported in optical densities (ODs). Other ELISAs performed in this thesis are commercially available and performed following the manufacturer's instructions (R&D systems).

5.3 Human Studies

5.3.1 Human lung tissue

Lung tissue is obtained from patients who undergo a surgical lung resection at Ghent University Hospital for solitary pulmonary tumours. Patients are excluded from the study when a treatment with neo-adjuvant chemotherapy, recent COPD exacerbation or pulmonary infection occurred. COPD diagnosis and severity was defined using pre-operative spirometry according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification [52]. Written informed consents were obtained from all subjects, according to the protocol approved by the medical ethical committee of Ghent University Hospital.

Tissue is collected by a pathologist at maximum distance from the lung lesion, showing no signs of retro-obstructive pneumonia or tumour invasion. Single cell suspension (Figure 19) is prepared for flow cytometric analyses (see 5.1.3.3).

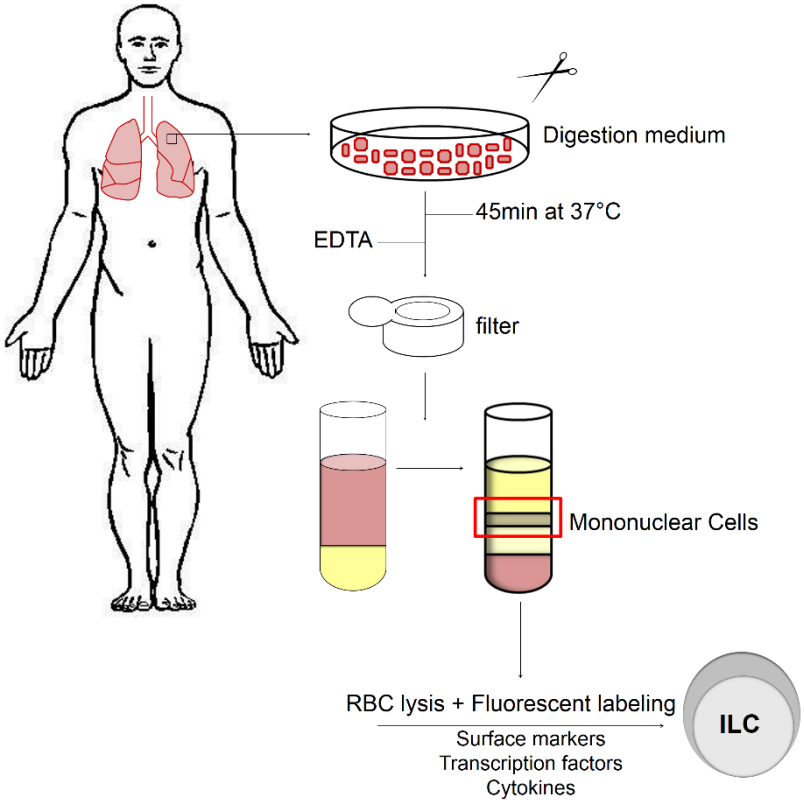


Figure 19: Protocol for the characterization of innate lymphoid cells in human lung tissue. Lung tissue is collected by a pathologist from patients who underwent a lobectomy. The lung tissue is cut into fine pieces and digested for 45 minutes at 37°C in digestion medium. Cells are resuspended in EDTA and filtered through a 40-µm cell strainer. Mononuclear cells are isolated using a ficoll gradient and subjected to red blood cell lysis. These cells are then fluorescently labelled for specific surface markers, transcription factors and cytokines to identify ILCs.

PART II: RESEARCH WORK

CHAPTER 6: RESEARCH OBJECTIVES

Asthma is a prevalent disorder of the conducting airways with a high socio-economic impact. Besides a genetic predisposition, also environmental exposures are important determining factors in the development of asthma. Multiple epidemiological and experimental studies provided evidence that inhalation of air pollutants greatly contributes to the induction as well as exacerbation of asthma. The underlying mechanisms remain however largely unknown. Considering that the current treatment options are predominantly symptomatic, novel treatments and therefore a better understanding in the asthma pathogenesis are urgently needed.

In a first part, we focused on **the role of the airway epithelium in pollutant-induced airway inflammation**, and more specifically investigated some specific epithelial-derived mediators, i.e. chemerin and IL-33.

- From our previous work it was clear that there is an important role for dendritic cells (DCs) at the frontline of diesel exhaust particles (DEPs)-modulated immune responses. The recruitment of these DCs towards the pulmonary tissue is regulated by diverse chemokine/cytokine interactions wherein the airway epithelium plays an important role. As chemerin, an epithelial-derived chemokine, induces cell chemotaxis of ChemR23 expressing cells, i.e. DCs and monocytes, towards the site of inflammation, **we hypothesized that chemerin/chemR23 signaling is involved in pollutant-induced inflammatory lung responses.** For this purpose, both the acute inflammatory effects of DEPs exposure and the enhancing effects of DEPs on allergic airway inflammation were evaluated in ChemR23 KO mice (Chapter 7).
- Inhalation of both DEPs and house dust mite (HDM) stimulates the airway epithelium to induce the production of innate pro-type2 cytokines, including IL-33. This IL-33 release is implicated in allergic airway inflammation as well as in functional DC maturation towards sole DEPs exposure. **Hence, we hypothesized that IL-33/ST2 signaling is crucial in DEPs-enhanced allergic airway inflammation.** To examine this, a recombinant decoy receptor (r-sST2) that neutralizes IL-33 activity was administrated to mice that were exposed to combined DEPs+HDM (Chapter 8).

In a second part, we were interested in **the contribution of ILCs in pollutant-aggravated inflammatory lung responses**

- Allergic asthma is typically considered as a T_H2-mediated disease. For many allergens an important contribution of ILC2s in allergic airway inflammation has been identified. Because DEPs aggravate the allergic airway inflammation, **we hypothesized a role**

for ILC2s in DEPs-enhanced allergic airway inflammation. In a first step, we investigated the presence and activation of ILC2s upon combined DEPs+HDM exposure. In a second step, we investigated whether the DEPs-enhanced responses were dependent on a GATA-3-mediated activation. Finally, the contribution of both ILC2s and T_H2 cells was evaluated in RAG2 KO and ILC2-deficient mice (Chapter 9).

- ILCs have been extensively studied over the years, as dysregulation of these cells can lead to the development of several inflammatory diseases. Whereas human ILC subsets have been predominantly studied in the intestines or skin, limited data was available concerning ILCs in the human respiratory system. **We hypothesized that all ILC subsets are present in human lung tissue and altered during lung pathology**. We first analysed the different ILC subsets in human lung tissue based on specific surface markers, transcriptional expression and cytokine production. Next, we tried to elucidate the relative abundance of ILCs in COPD (Chapter 10).

CHAPTER 7: PRO- AND ANTI-INFLAMMATORY ROLE OF CHEMR23 SIGNALING IN POLLUTANT-INDUCED INFLAMMATORY LUNG RESPONSES

Chemerin is an epithelial-derived chemokine that attracts ChemR23 expressing cells (i.e. monocytes, macrophages and DCs) to the site of inflammation. The role of the chemerin/chemR23 axis during respiratory diseases is however quite controversial. A pro-inflammatory role for ChemR23 signaling in response to cigarette smoke was already clearly demonstrated, whereas anti-inflammatory properties were observed during allergen exposure. In this study, we aimed to investigate the contribution of ChemR23 signaling in murine models of DEP-induced inflammatory lung responses.

Provoost S*, **De Grove KC***, Fraser GL, Lannoy VJ, Tournoy KG, Brusselle GG, Maes T, Joos GF. Pro- and Anti-Inflammatory Role of ChemR23 Signaling in Pollutant-Induced Inflammatory Lung Responses. Journal of Immunology. 2016; 196(4):1882-90. **(*Equal contribution)**

ABSTRACT

Rationale: Inhalation of traffic-related particulate matter (such as diesel exhaust particles, 'DEP') is associated with acute inflammatory responses in the lung and promotes the development and aggravation of allergic airway diseases. We previously demonstrated that exposure to DEP was associated with increased recruitment and maturation of monocytes and conventional dendritic cells (DC), resulting in T_H2 polarization. Monocytes and immature DC express the G-protein coupled receptor chemR23, which binds the chemoattractant chemerin.

Methods: Using chemR23 knockout (KO) and corresponding wild-type (WT) mice, we determined the role of chemR23 signaling 1) in response to acute exposure to DEP and 2) in response to DEP-enhanced house dust mite (HDM)-induced allergic airway inflammation.

Results: Exposure to DEP alone, as well as combined exposure to DEP + HDM, elevated the levels of chemerin in the bronchoalveolar lavage fluid of WT mice. In response to acute exposure to DEP, monocytes and monocyte-derived DC accumulated in the lungs of WT mice but this response was significantly attenuated in chemR23 KO mice. Concomitant exposure to DEP + HDM resulted in allergic airway inflammation with increased eosinophilia, goblet cell metaplasia and T_H2 cytokine production in WT mice, which was further enhanced in chemR23 KO mice.

Conclusion: In conclusion, we demonstrated an opposing role for chemR23 signaling depending on the context of DEP-induced inflammation: the chemR23 axis showed pro-inflammatory properties in a model of DEP-induced acute lung inflammation, in contrast to anti-inflammatory effects in a model of DEP-enhanced allergic airway inflammation.

INTRODUCTION

It is well accepted that inhalation of traffic-related particulate matter, of which diesel exhaust particles (DEP) are a main component, is associated with acute inflammatory responses in the lung. In addition, traffic-related particulate matter can contribute to new-onset asthma as well as to exacerbations of pre-existing asthma [60]. Experimental studies in mice showed that exposure to DEP can enhance allergic airway responses, including eosinophilia, goblet cell metaplasia and T_H2 production [23]. In addition, in controlled human exposure studies, combined DEP + allergen exposure increased allergen-specific immunoglobulin levels and induced a T_H2 cytokine pattern [23, 100]. The mechanistic basis of the inflammatory response in the lung to DEP inhalation as well as the adjuvant response to DEP on allergen-induced airway inflammation remain incompletely known.

Using a mouse model of DEP-induced acute lung inflammation, we previously demonstrated that exposure to DEP was associated with increased expression of pro-inflammatory cytokines/chemokines and with the accumulation of neutrophils and monocytes in lung tissue [91]. Furthermore, we showed that DEP had a great impact on the biology of conventional dendritic cells (DC) [94], which are crucial in the induction of asthma. We demonstrated that exposure to DEP induced the recruitment of monocytes and DC towards the lung via the G-protein coupled receptors CCR2 and CCR6, increased DC maturation and enhanced DC-induced allergen transport towards the draining lymph nodes, resulting in T_H2 polarization. This modulation of DC function could be the mechanistic basis underlying DEP-enhanced allergic airway responses [94, 95].

ChemR23 (also known as chemokine-like receptor 1, CMKLR1) is a seven-transmembrane G-protein coupled receptor that is expressed on monocytes, macrophages, natural killer cells, conventional and plasmacytoid DC in humans and mice [128, 282, 283]. Its ligand, chemerin, is secreted as a weakly active precursor protein (i.e. prochemerin) that is converted into bioactive chemerin by proteolytic cleavage of its C-terminal [284]. This maturation step is mediated by extracellular proteases released by activated macrophages, mast cells and neutrophils [129, 285, 286]. In addition to chemerin, resolvin E1 (RvE1, an anti-inflammatory lipid, derived from the omega-3 fatty acid eicosapentaenoic acid) is reported as a second putative ligand of chemR23 [134].

Intriguingly, the role of the chemerin/chemR23 axis in inflammation is controversial and seems to have pro- and anti-inflammatory properties depending on the model that is investigated. Previous work from our lab demonstrated that exposure to cigarette smoke was associated with increased chemerin levels in the bronchoalveolar lavage fluid (BALF). Moreover, chemR23

knockout (KO) mice were almost completely protected against cigarette smoke-induced lung inflammation [120]. In contrast to this pro-inflammatory role of chemR23 signaling in response to cigarette smoke, chemR23 KO mice showed an increased inflammatory response in a LPS-induced model of acute lung injury [131] and were more susceptible to viral pneumonia [136]. In addition, intranasal administration of exogenous chemerin was associated with attenuated allergic airway inflammation and airway hyperresponsiveness [121].

Here, we studied the role of chemR23 signaling in the context of DEP-induced lung responses. First, we examined the chemR23 axis in the murine model of DEP-induced lung inflammation. Next, we investigated the contribution of chemR23 signaling to the adjuvant capacity of DEP in enhancing allergic airway inflammation, using a model of concomitant exposure to DEP and a clinically relevant allergen (i.e. house dust mite, HDM). We demonstrated that the functional role of chemR23 signaling is dependent upon the background inflammatory conditions, which explains the existing controversy in the literature.

MATERIAL AND METHODS

Animals

Female C57BL/6 mice (6-8 weeks old) were purchased from Harlan. Female ChemR23 KO mice (6-9 weeks old) and corresponding C57BL/6 WT mice (6-9 weeks old) were obtained from Deltagen and bred in our animalarium. All *in vivo* manipulations were reviewed and approved by our local ethical committee (Animal Ethical Committee of the Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium).

DEP and HDM administrations

DEP (SRM 2975) was obtained from the National Institute for Standards and Technology. For the mouse model of DEP-induced acute lung inflammation, DEP were suspended in saline (B. Braun Melsungen) containing 0.05% Tween 80 (Invitrogen). Mice were anesthetized with an intraperitoneal ketamine/xylazine injection (Ketamine 1000 CEVA (70 mg/kg), Ceva Sante Animale; Rompun 2% (7 mg/kg), Bayer) prior to instillation. The anesthetized mice were intratracheally instilled with 50 µl saline or DEP solution (i.e. 100 µg) on day 1, 4 and 7. On day 9, the animals were sacrificed by a lethal dose of intraperitoneal pentobarbital (Ceva Sante Animale) (4-6). For the mouse model of DEP-enhanced allergic airway inflammation, HDM (Dermatophagoides pteronyssinus; Greer Laboratories) was suspended in saline. Mice were anesthetized with isoflurane (Abbott Laboratories) prior to instillation. The anesthetized mice were intranasally instilled with 50 µl saline, DEP (i.e. 25 µg), HDM (i.e. 1 µg) or combined DEP plus HDM solution on day 1, 8 and 15. On day 17, the animals were sacrificed by a lethal dose of intraperitoneal pentobarbital (Ceva Sante Animale). Tween 80 (0.05%) was used as a vehicle in both murine models and in all conditions.

BALF

A tracheal cannula was inserted and BALF was recovered by instillation of 3 x 300 µl HBSS w/o Ca2+ or Mg2+ (BioWittaker) supplemented with 1 % BSA (for cytokine and chemokine measurements; Sigma-Aldrich) and 6 x 500 µl HBSS w/o Ca2+ or Mg2+ supplemented with 0.6 mM sodium EDTA (Sigma-Aldrich). The lavage fractions were pooled and total cell counts were performed using a Bürcker chamber (Brand GMBH).

Lung and mediastinal LN single cell suspensions

Pulmonary circulation was rinsed with saline, supplemented with EDTA, to remove the intravascular pool of cells. Lungs or mediastinal LN were minced and incubated in digestion medium (RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamine, 0.05 mM 2-mercaptomethanol (all Invitrogen), 100 U/ml penicillin - 100 µg/ml streptomycin (Sigma-Aldrich), 1 mg/ml collagenase type 2 (Worthington Biochemical) and 0.02 mg/ml DNase I (grade II from bovine pancreas; Boehringer Ingelheim)) for 45 minutes at 37°C and 5% CO2. Red blood cells were lysed using ammonium chloride buffer. Cell counting was performed with a Z2TM coulter counter (Beckman Coulter).

Flow cytometry

All staining procedures were performed in PBS w/o Ca2+ or Mg2+ containing 5 mM EDTA and 1% BSA. To minimize non-specific bindings, BALF cells and lung single cell suspensions were incubated with anti-CD16/CD32 (clone 2.4G2). Cells were labeled with combinations of CD11c (HL3), MHCII (2G9), CD11b (M1/70), Ly6C (AL-21), Ly6G (1A8), Siglec-F (e50-2440), CD4 (GK1.5), CD8 (53-6.7), CD69 (H1.2F3), CD3 (145-2C11) (all BD Biosciences). Data acquisition was performed on a FACSCaliburTM flow cytometer running CELLQuestTM software or on a LSRFortessaTM cell analyser running FACSDivaTM software (BD Biosciences). FlowJo software was used for data analysis.

Cell culture

Mediastinal LN cells were cultured in culture medium (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 0.05 mM 2-mercaptomethanol (all Invitrogen), 100 U/ml penicillin - 100 µg/ml streptomycin (Sigma-Aldrich)) either alone or supplemented with 3.75 µg/well HDM in round bottom 96-well plates, and incubated in a humidified 37°C incubator with 5% CO2. After 5 days, supernatants was harvested for protein measurements.

Histology

Lungs were fixed in 4% paraformaldehyde and embedded in paraffin (Klinipath). 3 µm transversal sections were cut, treated with Ultra V Block (Thermo Scientific) and incubated with polyclonal anti-mouse chemerin antibody (R&D Systems). The goat HRP-Polymer Kit (Biocare Medical) and diaminobenzidine (DakoCytomation) were used for detection. Prochemerin staining was quantified within the airway epithelium in a marked area between the airway lumen and the basement membrane, using KS400 Software (Zeiss) [120]. The area with positive staining for prochemerin was normalized to the basement membrane perimenter (Pbm) (nomenclature described in [287]). All airways with Pbm >2000 µm were excluded. For the visualisation of eosinophils or goblet cells, lungs were stained with Congo-Red or Periodic Acid Schiff (Klinipath). Quantification was performed (KS400 software) in all airways with Pbm between 800 and 2000 µm.

Protein measurements

Chemerin, CCL2, CCL20, interleukin (IL)-4, IL-5 and IL-13 levels in BALF or mediastinal LN culture supernatant were measured using commercially available ELISA kits (R&D Systems). Ig were determined on serum that was collected by retro-orbital bleeding. Total IgE was measured using coated plates and biotinylated polyclonal rabbit anti-mouse IgE (S. Florquin, Université libre de Bruxelles). For detection of HDM-specific IgG1, plates were coated with HDM extract. Serum was added, followed by a HRP-conjugated polyclonal goat anti-mouse IgG antibody (Bethyl Laboratories). Ig values were reported in optical densities (OD).

RT-PCR

Hematopoietic ($CD45^+$) cells and non-hematopoietic ($CD45^-$) lung cells were sorted using an OctoMACS separator and CD45 MicroBeads (according to manufacturer's instructions; Miltenyi Biotec). The sorted populations showed >95 % purity (data not shown). Next, RNA extraction was performed (miRNeasy Mini Kit; Qiagen) and cDNA (Transcriptor First Strand cDNA synthesis kit; Roche Diagnostics) was obtained following manufacturer's instructions. SYBR Green-based RT-PCR reactions (LightCycler 480 SYBR Green I Master; Roche) were performed using a LightCycler 96 system (Roche). Chemerin primer sequences were previously described in (15). Data was processed using the standard curve method. Expression of chemerin mRNA was normalized based on the expression of two reference genes (GADPH and HPRT).

Statistical analysis

Statistical analysis was performed with SPSS, version 22.0. Groups were compared using nonparametric tests (Kruskall-Wallis and Mann-Whitney U) according to standard statistical criteria. Reported values were expressed as mean ± SEM. Values of $p < 0.05$ were regarded as significant.

RESULTS

Role of chemR23 signaling in a model of DEP-induced lung inflammation

Chemerin levels in BALF are increased in WT mice exposed to DEP

We previously identified airway epithelial cells as the major source of (pro)-chemerin in lung tissue [120]. To examine the localisation and expression of (pro)-chemerin in response the DEP, we exposed WT mice to saline or DEP (experimental protocol in **Fig. 1A**), sorted hematopoietic ($CD45^+$) versus structural non-hematopoietic ($CD45^-$) cells from lung tissue, and examined the chemerin mRNA expression. Chemerin was mainly expressed in the $CD45^-$ cell population, whereas minimal chemerin mRNA expression was observed in the $CD45^+$ cell population. Chemerin mRNA expression in $CD45^-$ cells significantly increased in response to exposure to DEP **(Fig. 1B)**. Using immunohistochemistry, we confirmed that the epithelium was the predominant source of (pro)chemerin in the lung. We quantified the (pro)chemerin staining and observed that exposure to DEP was associated with decreased (pro)chemerin staining in the epithelium when compared to control mice **(Fig. 1C)**. To assess whether this was due to increased secretion, we determined (pro)chemerin levels in BALF using ELISA, and found that exposure to DEP elevated the secreted levels of (pro)chemerin **(Fig. 1D)**. RvE1 is a second putative ligand of chemR23 [134]. Using ELISA, we assessed the RvE1 levels in the BALF and found that these were decreased in response to DEP exposure **(Fig 1E).**

ChemR23 is required for DEP-induced monocyte and alveolar DC recruitment

Chemerin/chemR23 signaling can mediate the recruitment of monocytes and (immature) DC [284]. To assess the contribution of the chemR23 pathway to the accumulation of monocytes and DC in response to DEP, we exposed WT and chemR23 KO mice to saline or DEP. Upon exposure to DEP, the number of total BALF cells increased in WT mice. This increase was smaller in chemR23 KO mice **(Fig. 2A)**. Exposure to DEP was associated with an accumulation of monocytes in the BALF and lung, and monocyte-derived DC in lung of WT mice, which was severely reduced or absent in chemR23 KO mice **(Fig. 2B-D)**. In addition, DEP exposure increased the number of alveolar DC and their maturation, as demonstrated by increased expression of CD86. These responses were significantly attenuated in chemR23 KO mice compared to WT mice **(Fig. 2E and F)**. In contrast, the DEP-induced increase in lung $CD11b^+$ conventional DC and neutrophils in BALF and lung were similar between WT and chemR23 KO mice **(Fig. 2G-I)**. Lung macrophage counts were not affected by DEP exposure and did not differ between WT and chemR23 KO mice **(Fig. 2J)**.

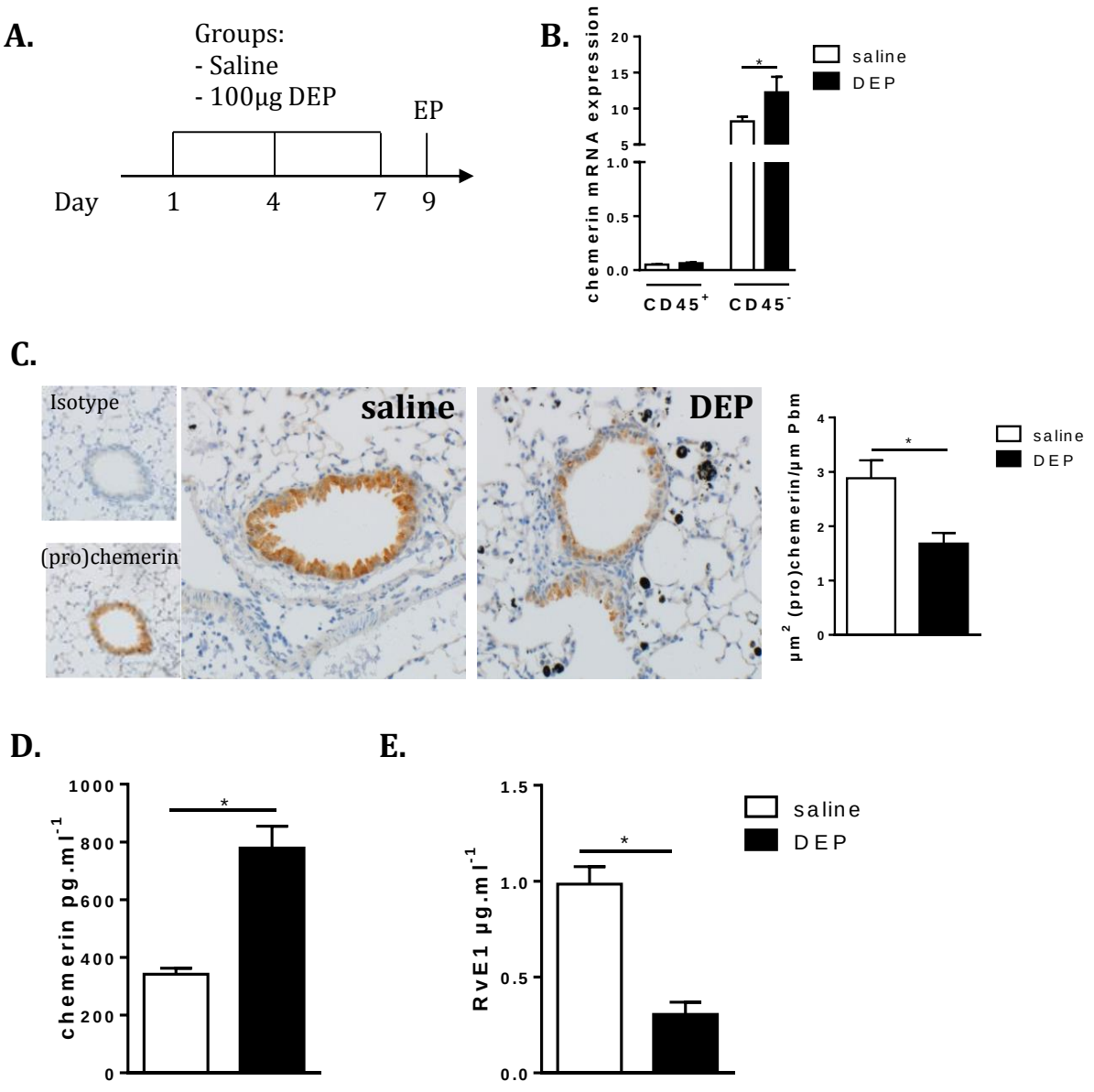


FIGURE 1: Chemerin levels in BALF are increased in WT mice exposed to DEP. Mice were intratracheally exposed to saline (white bar) or 100 µg DEP (black bar). **A,** schematic overview of the model of DEP-induced acute lung inflammation. EP = endpoints. **B,** chemerin mRNA expression in hematopoietic ($CD45^+$) and structural non-hematopoietic ($CD45^-$) lung cells was determined using RT-PCR. **C,** photomicrographs of prochemerin staining in the airway epithelium of mice that were exposed to saline or DEP and quantification of prochemerin staining. **D-E,** chemerin **(D)** and RvE1 **(E)** RvE1 protein levels in BALF were determined by ELISA. Results are expressed as mean ± SEM. n = 8 mice per group. *$p < 0.05$.

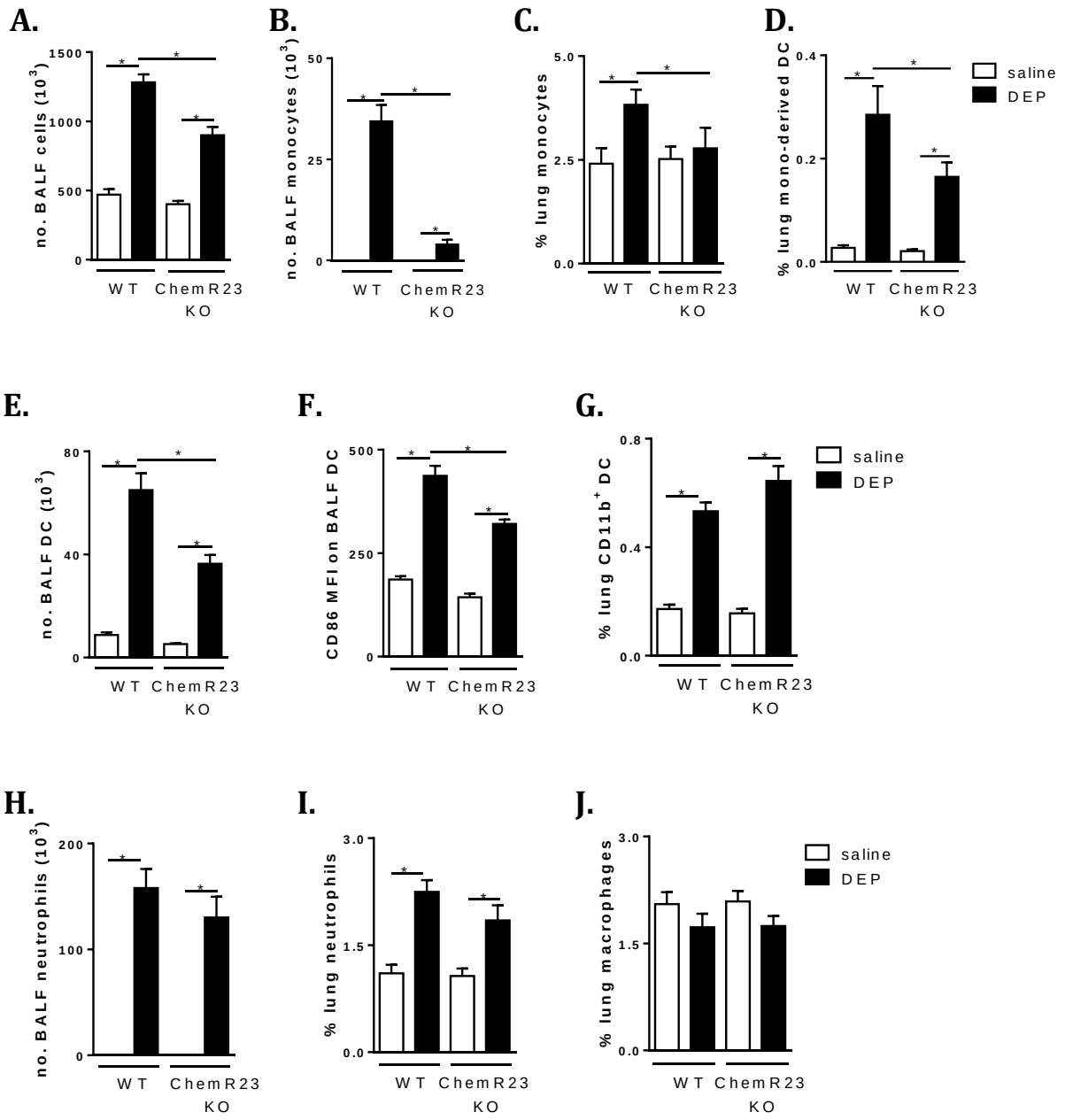


FIGURE 2: ChemR23 is required for DEP-induced monocyte and alveolar DC recruitment. WT and chemR23 KO mice were exposed to saline (white bars) or DEP (black bars). **A,** total cell number in BALF. **B-J,** monocytes numbers in BALF ($CD11c^{low}$, $CD11b^{+}$, $Ly6C^{+}$ and $Ly6G^{-}$) **(B)**; % monocytes in lung tissue ($CD11c^{low}$, $CD11b^{+}$, $Ly6C^{+}$ and $Ly6G^{-}$) **(C)**; % lung monocyte-derived DC ($CD11c^{high}$, low autofluorescent, $CD11b^{+}$ and $Ly6C^{+}$) **(D)**; DC numbers in BALF ($CD11c^{high}$, low autofluorescent and $MHCII^{+}$) **(E)**; mean fluorescence intensity (MFI) of CD86 on alveolar DC **(F)**; % lung $CD11b^{+}$ conventional DC ($CD11c^{high}$, low autofluorescent, $CD11b^{+}$ and $Ly6C^{-}$) **(G)**; neutrophils numbers in BALF ($CD11c^{low}$, $CD11b^{+}$, $Ly6C^{+}$ and $Ly6G^{+}$) **(H)**; % lung neutrophils ($CD11c^{low}$, $CD11b^{+}$, $Ly6C^{+}$ and $Ly6G^{+}$) **(I)**; and % lung macrophages ($CD11c^{high}$, high autofluorescent) **(J)** were determined by flow cytometry. Results are expressed as mean ± SEM. n = 7-8 mice per group. *$p < 0.05$. Representative flow cytometric density plots and gating strategy can be found in the material and methods section (Figure 15, p. 54).

ChemR23 is required for DEP-induced CCL2 and CCL20 production

CCL2 and CCL20 are important chemokines for the DEP-induced recruitment of monocytes and monocyte-derived DC [95]. DEP exposure elevated the levels of CCL2 and CCL20 in the BALF in both WT and chemR23 KO mice. However, the levels of these chemokines were significantly reduced in DEP-exposed chemR23 KO mice **(Fig. 3A and B)**. The level of the chemR23 ligand, chemerin, in BALF was elevated in WT mice that received DEP as described above. Independent of the exposure, all chemR23 KO mice had increased chemerin levels when compared to WT controls **(Fig. 3C)**.

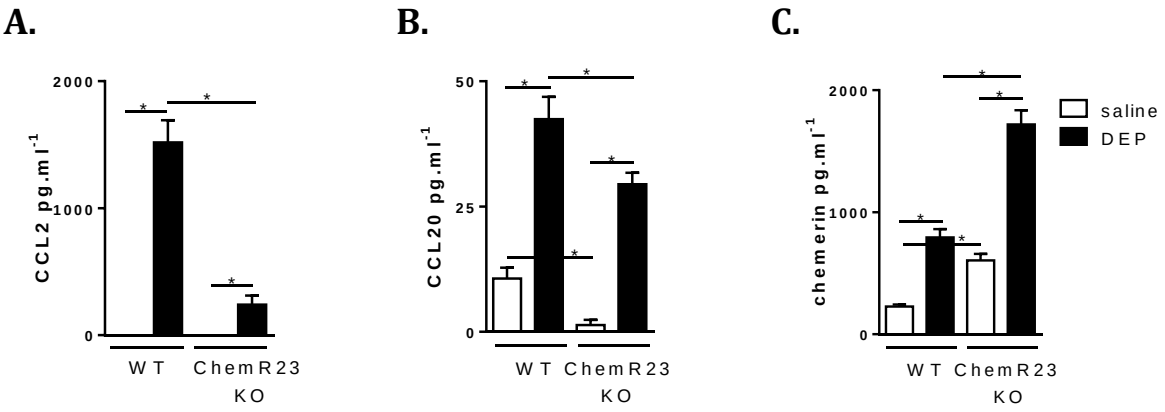


FIGURE 3: ChemR23 is required for DEP-induced CCL2 and CCL20 production. WT and chemR23 KO mice were exposed to saline (white bars) or DEP (black bars). **A-C,** CCL2 **(A)**; CCL20 **(B)**; and chemerin **(C)** protein levels in BALF were determined by ELISA. Results are expressed as mean ± SEM. n = 7-8 mice per group. *$p < 0.05$.

Role of chemR23 signaling in a model of DEP-enhanced allergic airway inflammation

Chemerin levels are increased in WT mice co-exposed to DEP + HDM

Exposure to DEP promotes sensitization towards co-inhaled allergens and aggravates asthma [23]. To investigate the molecular mechanisms, we set up a model wherein we exposed mice to saline, DEP or HDM alone, or combined DEP and HDM (experimental protocol in **Fig. 4A**). In order to examine the adjuvant capacity of DEP optimally, we administered low doses of DEP and HDM, that elicited almost no inflammatory or allergic response on their own. In this model, the chemerin levels in BALF were elevated in WT mice concomitantly exposed to DEP + HDM, compared to all control groups **(Fig. 4B)**. Levels of RvE1 were decreased in WT mice exposed to DEP or HDM alone, or combined DEP + HDM, when compared to saline-exposed WT mice **(Fig. 4C)**.

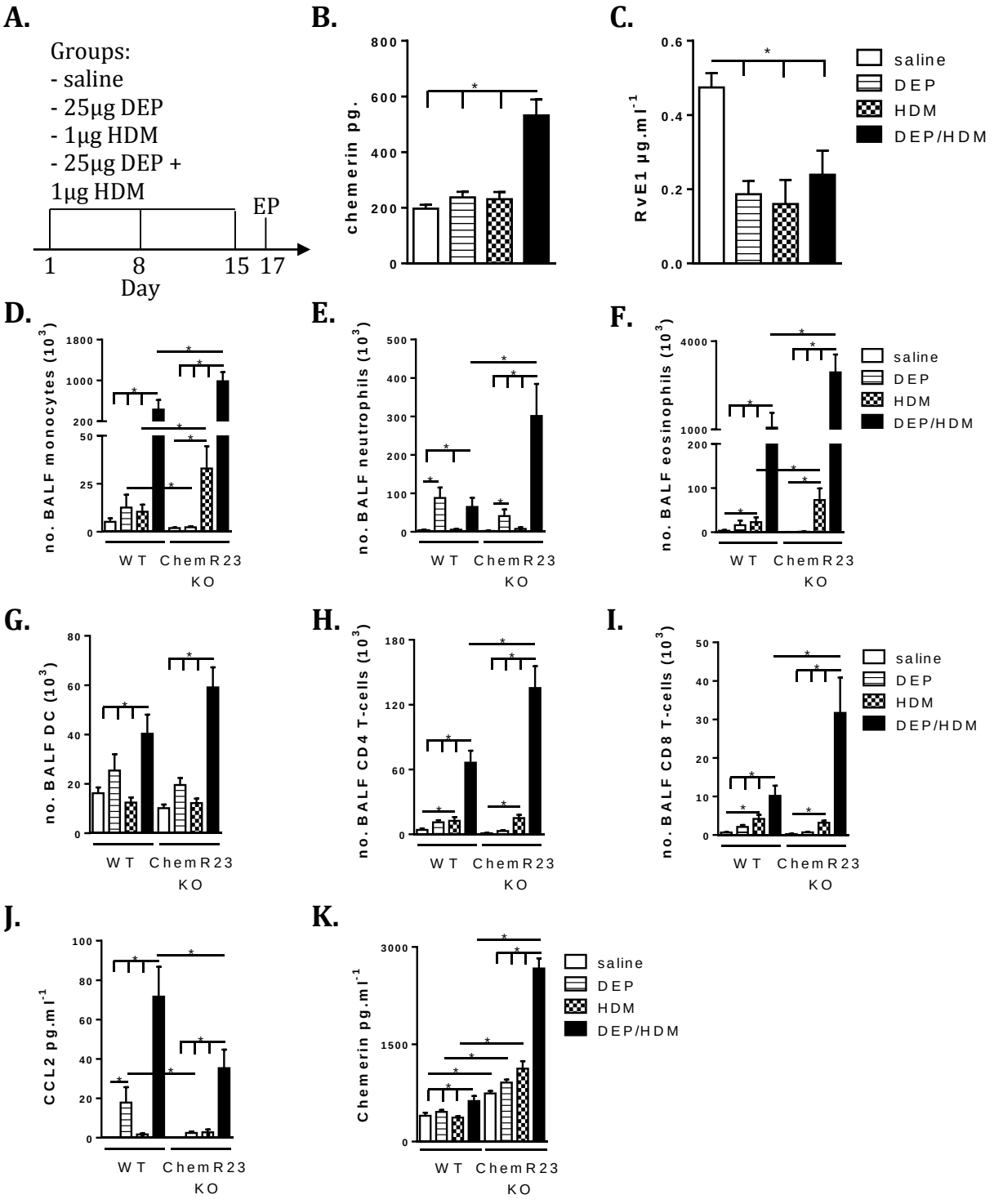


FIGURE 4: Absence of chemR23 aggravates allergic responses in BALF upon combined DEP + HDM exposure. WT and chemR23 KO mice were intranasally exposed to saline (white bar), 25 µg DEP (striped bar), 1µg HDM (checked bar) or 25 µg DEP + 1 µg HDM (black bar). **A,** schematic overview of the model of DEP-enhanced allergic airway inflammation. EP = endpoints. **B-C,** chemerin **(B)** and RvE1 **(C)** protein levels in BALF were determined by ELISA. **D-I,** monocyteS numbers ($CD11c^{low}$, $CD11b^{+}$, $Ly6C^{+}$ and $Ly6G^{-}$) **(D)**; neutrophils numbers ($CD11c^{low}$, $CD11b^{+}$, $Ly6C^{+}$ and $Ly6G^{+}$) **(E)**; eosinophils **(F)**; DC numbers ($CD11c^{high}$, low autofluorescent and $MHCII^{+}$) **(G)**; $CD4^{+}$ T-cell numbers ($CD3^{+}$, $CD8^{-}$ and $CD4^{+}$) **(H)**; and $CD8^{+}$ T-cell numbers ($CD3^{+}$, $CD4^{-}$ and $CD8^{+}$) **(I)** in BALF were determined by flow cytometry, except eosinophils which were determined on cytospins. **J-K,** CCL2 **(J)**; and chemerin **(K)** protein levels in BALF were determined by ELISA. Results are expressed as mean ± SEM. n = 7-12 mice per group. *p < 0.05. Lines with branches represent significant differences between DEP + HDM versus saline, DEP alone or HDM alone, except in **(C)** were the line with braches represents differences between saline versus DEP alone, HDM alone and DEP +HDM.

Absence of chemR23 aggravates allergic responses in BALF upon combined DEP + HDM exposure

To elucidate the role of chemR23 signaling in DEP-enhanced allergic airway inflammation, we exposed WT and chemR23 KO mice to saline, DEP or HDM alone, or combined DEP + HDM. In this protocol, exposure to sole DEP in WT mice was associated with marginal increases in monocytes and neutrophils in BALF, when compared to WT mice that were exposed to saline **(Fig. 4D and E)**. Sole HDM slightly increased the amount of BALF eosinophils, $CD4^+$ T-cells and $CD8^+$ T-cells compared to the saline WT group **(Fig. 4F, H and I)**. In contrast, concomitant exposure to DEP + HDM greatly enhanced the inflammatory and allergic response in WT mice, with increased numbers of monocytes, eosinophils, DC, $CD4^+$ T-cells and $CD8^+$ T cells in the BALF, when compared to the three WT control groups. This was further increased in chemR23 KO mice that were exposed to combined DEP + HDM **(Fig. 4D, F-I)**. Of interest, chemR23 KO mice seemed more susceptible to allergic airway inflammation, since chemR23 KO receiving sole HDM had increased monocytes and eosinophils when compared to HDM-exposed WT mice **(Fig. 4D and F)**. In line with the data from the model of DEP-induced lung inflammation, the number of BALF monocytes were attenuated in chemR23 KO mice that were exposed to DEP alone, when compared to DEP-exposed WT mice **(Fig. 4D)**. Levels of CCL2 in BALF were elevated in WT mice exposed to DEP, and increased further in mice that received concomitant DEP + HDM. In contrast, chemR23 KO mice had decreased CCL2 levels when compared with their WT controls **(Fig. 4J)**. The levels of chemerin were increased in mice that were co-exposed to DEP + HDM as described above. Independent of the exposure, all chemR23 KO had increased chemerin levels when compared to WT controls **(Fig. 4K)**.

Absence of chemR23 aggravates allergic responses in lung upon combined DEP + HDM exposure

Similarly to the data in the BALF, eosinophils, DC and $CD4^+$ T-cells accumulated in the lungs of mice that were concomitantly exposed to DEP + HDM, when compared to WT control groups. Again, this response was further increased in ChemR23 KO mice that received combined DEP + HDM **(Fig. 5A-C)**. ChemR23 KO mice that received sole HDM had slightly increased eosinophils and DC when compared to HDM-exposed WT mice **(Fig. 5A and B)**. Histological analysis revealed peribronchial eosinophilic inflammation and increased goblet cells in WT mice that were exposed to combined DEP + HDM. In chemR23 KO mice that received DEP + HDM, this was further increased **(Fig. 5D and E respectively)**. While low dose HDM on its own had no significant effect on eosinophilia or goblet cell metaplasia in WT mice, numbers of peribronchial eosinophils and goblet cells were elevated in chemR23 KO that received sole HDM **(Fig. 5D and E)**.

Absence of chemR23 increases type 2 cytokine production upon combined DEP + HDM exposure

To assess type 2 cytokine production in the model of DEP-enhanced allergic inflammation, mediastinal LN were cultured with HDM and analysed for cytokine production. LN cells from WT mice that were previously exposed to saline, sole DEP or low dose HDM showed no production of type 2 cytokines. However, combined exposure to DEP + HDM was associated with increased production of IL-4, IL-5 and IL-13 in WT mice. Production of type 2 cytokines was amplified in chemR23 KO mice that received DEP + HDM **(Fig. 6 A-C)**. Exposure to HDM alone was also associated with increased IL-5 and IL-13 levels in chemR23 KO mice in comparison with HDM-exposed WT mice **(Fig. 6 B-C)**. Total serum IgE and HDM-specific IgG1 were elevated in WT mice that received combined DEP + HDM. The levels tended to increase in chemR23 KO mice that were exposed to DEP + HDM **(Fig. 6D and E)**.

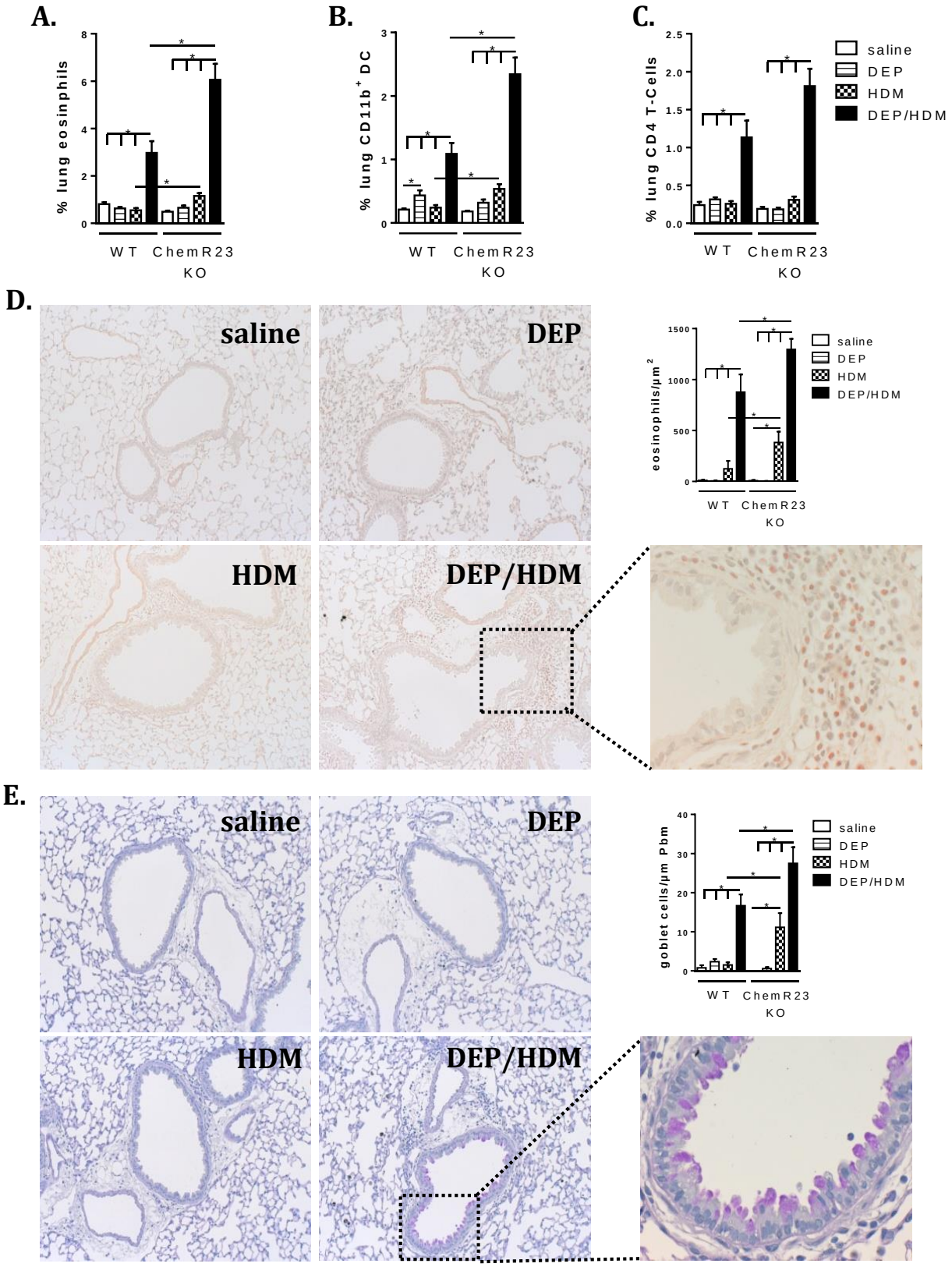


FIGURE 5: Absence of chemR23 aggravates allergic responses in lung upon combined DEP + HDM exposure. WT and chemR23 KO mice were exposed to saline (white bars), DEP (striped bars), HDM (checked bars) or DEP + HDM (black bars). **A-C,** % eosinophils (CD11c^{low}, CD11b^{+} and Siglec-F^{+}) **(A)**; % CD11b+ conventional DC (CD11c^{high}, low autofluorescent, CD11b^{+} and MHCII^{+}) **(B)**; and % CD4^{+} T-cells (CD3^{+}, CD8^{-}, CD4^{+} and CD69^{+}) **(C)** in lung tissue were determined by flow cytometry. Representative flow cytometric density plots and gating strategy can be found in the materials & methods section (Figure 15, p.54). **D-E,** photomicrographs and quantification of Congo Red **(D)** and Periodic Acid Schiff **(E)** stained sections of the airways of mice that were exposed to saline, DEP, HDM or DEP + HDM. Representative photomicrographs from WT mice are shown. Results are expressed as mean ± SEM. n = 7-12 mice per group. *p < 0.05. Lines with branches represent significant differences between DEP + HDM versus saline, DEP alone or HDM alone.

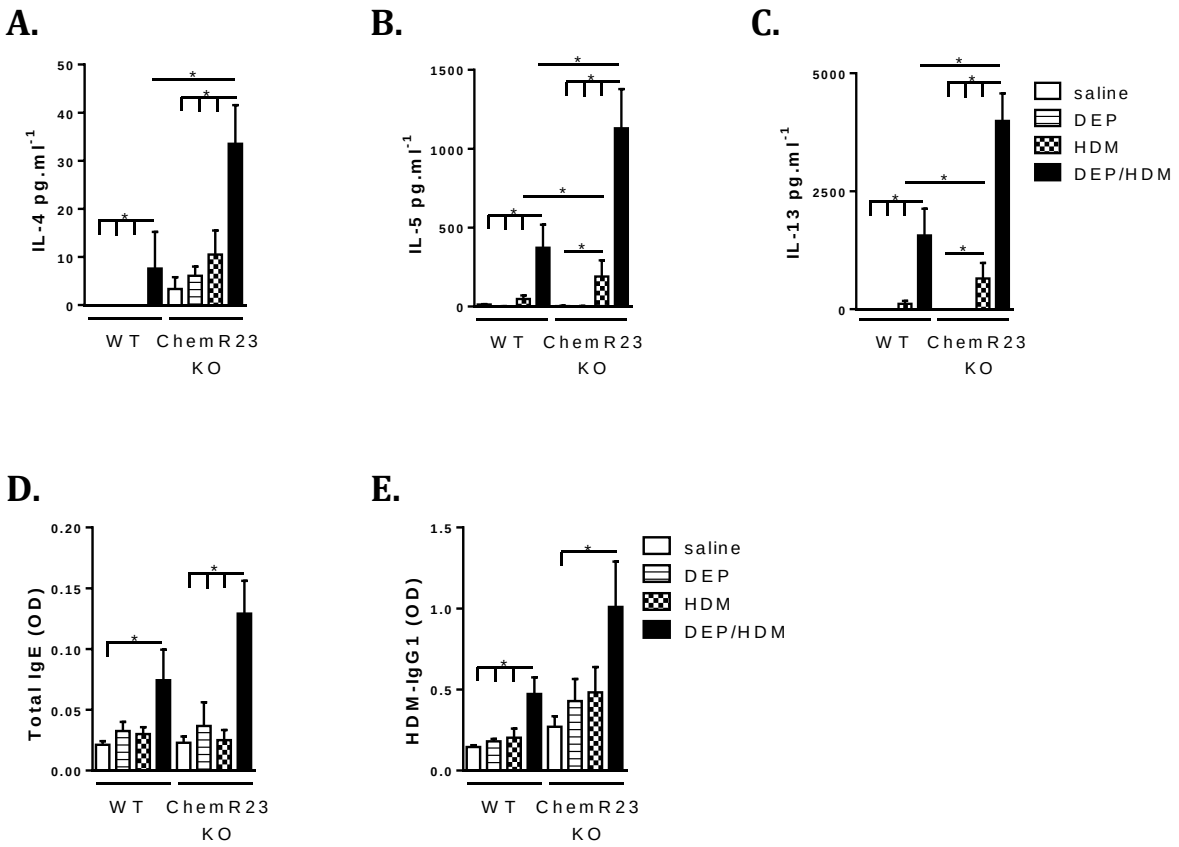


FIGURE 6: Absence of chemR23 increases type 2 cytokine production and immunoglobulins upon combined DEP + HDM exposure. WT and chemR23 KO mice were exposed to saline (white bars), DEP (striped bars), HDM (checked bars) or DEP + HDM (black bars). **A-C,** IL-4 **(A)**; IL-5 **(B)**; and IL-13 **(C)** protein levels in the supernatant of HDM-restimulated LN cells were determined by ELISA. **D-E,** Total IgE **(E)**; and HDM-specific IgG1 **(E)** in serum were determined by ELISA. Results are expressed as mean. n = 7-12 mice per group. *$p < 0.05$. Lines with branches represent significant differences between DEP + HDM versus saline, DEP alone or HDM alone.

DISCUSSION

In this study, we showed that the response to modulation of the chemerin/chemR23 axis is contingent upon the specific conditions of DEP-induced airway inflammation. In the model of DEP-induced lung inflammation, chemR23 KO mice had decreased numbers of monocytes and DC upon DEP-exposure compared to WT mice, suggesting a pro-inflammatory role for chemR23 signaling. In contrast, in the model of DEP-enhanced allergic airway inflammation, chemR23 KO mice had increased lung eosinophilia, goblet cell metaplasia and T_H2 cytokine production in response to DEP + HDM compared to WT mice, suggesting an anti-inflammatory role for the chemR23 axis.

In the lung, epithelial cells are the first to encounter inhaled particles and allergens. In response to immunostimulatory antigens, the epithelium can release cytokines and chemokines that direct the recruitment and/or activation of innate and adaptive immune cells [288]. Upon exposure to DEP, we observed decreased (pro)chemerin protein staining in the airway epithelium, which was associated with an increased (pro)chemerin release in the BALF. Similar observations were previously made in our model of cigarette-smoke induced inflammation [120], suggesting that exposure to pollutants can trigger the epithelium to release (pro)-chemerin in the alveolar lumen. In addition, we demonstrated a synergistic response between DEP and HDM to induce (pro)-chemerin secretion in the BALF.

After proteolytic activation, chemerin attracts chemR23-expressing immune cells, including monocytes and monocyte-derived DC [284]. In chemR23 KO mice the accumulation of pulmonary monocytes and monocyte-derived DC was decreased in the model of DEP-induced lung inflammation, suggesting a pro-inflammatory role for chemR23 signaling, which is similar to previous observations reported by our group in a model of cigarette smoke-induced inflammation [120].

Monocyte-derived DC are known to stimulate T_H2 immunity in response to inhaled allergens [95, 289]. Since we observed decreased monocyte and monocyte-derived DC numbers in chemR23 KO mice that were exposed to DEP alone, we were interested in the role of chemR23 signaling in a model of allergic inflammation enhanced by DEP. Intriguingly, we found an increased allergic airway inflammation in chemR23 KO mice that were exposed to concomitant DEP + HDM, suggesting an anti-inflammatory role of the chemR23 axis in DEP-enhanced allergic airway inflammation. Despite the modest response towards HDM alone in our model, chemR23 KO mice were also more susceptible to (exclusively) HDM-induced allergic airway inflammation, with enhanced eosinophilia and T_H2 cytokine production compared to WT mice. Interestingly, in independent models of HDM- or OVA-induced airway inflammation, exogenously administered chemerin was reported to be associated with an anti-inflammatory response [121, 290],

suggesting that chemerin mediates its anti-inflammatory properties in models of allergic airway inflammation by signaling via chemR23. The chemerin/chemR23 axis was also found to mediate anti-inflammatory responses in models of viral pneumonia and LPS-induced lung inflammation [131, 136].

Although a direct effect of active chemerin on monocytes has been described [284], it is of interest that chemR23 KO mice had impaired levels of CCL2 and CCL20. We previously showed that monocyte and monocyte-derived DC recruitment in response to DEP is attenuated in mice deficient for CCR2 and CCR6, which are the receptors for CCL2 and CCL20 respectively [95]. Possibly, the reduced monocytic response in DEP-exposed chemR23 KO mice is therefore a consequence from the reduced levels of CCL2 and CCL20, rather than representing a direct role for the chemR23 axis in monocyte recruitment, per se. Moreover, the reduced CCL2 and CCL20 levels in the chemR23 KO mice could suggest that chemR23 signaling is required for the upregulation in CCL2 and CCL20 in response to DEP. The fact that chemR23 KO mice receiving concomitant DEP + HDM also had decreased CCL2 levels compared to WT mice supports the suggestion that chemR23 signaling modulates the production of CCL2. Furthermore, it suggests that the enhanced allergic inflammation and monocyte recruitment that is elicited in the lungs of chemR23 KO mice by DEP + HDM is independent of CCL2, which is in contrast to the findings of a previous report [121].

The underlying basis of these opposing activities of chemerin/chemR23 signaling remains unclear [132]. Various proteases can process prochemerin to bioactive chemerin. Thus, depending on the proteases that are present in the microenvironment, diverse chemerin fragments may be produced with distinct pharmacological properties. Serine proteases (i.e. neutrophil elastase and cathepsin G) that are released upon neutrophil degranulation generate chemerin-157 and chemerin-156 (i.e. prochemerin lacking the last six and seven amino acids, respectively) that are associated with the recruitment of chemR23-expressing antigen presenting cells [129, 284]. Since exposure to DEP and cigarette smoke is associated with neutrophil recruitment to the lung and increased expression of neutrophil-derived proteases [91, 120], one can speculate that inhalation of air pollutants promotes a microenvironment that generates pro-inflammatory peptides of chemerin. On the other hand, mast cell chymase can convert these chemerin-157 and -156 peptides into chemerin-154, which is associated with anti-inflammatory actions on chemR23 [285, 286]. Thus, chemerin may be cleaved into diverse anti-inflammatory peptides in response to allergic airway inflammation wherein the activation of mast cells is a prominent feature. A limitation of our study is that we could not distinguish between prochemerin and the bioactive isoforms of chemerin in the 2 models. To our knowledge, there are no commercially available antibodies that efficiently discriminate between prochemerin or the various chemerin isoforms.

Nevertheless, such tools are necessary to get more insights in the complex biology of chemerin signaling, since prochemerin and the multiple chemerin isoforms can act as antagonists for each other's activity, and can all compete for chemR23 binding and ultimate biological response [291]. To overcome this, we have attempted to measure chemerin bioactivity in our samples using an aequorin-based calcium assay [136] (data not shown). Unfortunately, the chemerin levels were too low to detect any bioactivity, yet this does not exclude that local lung chemerin levels contribute to leukocyte recruitment.

Recently, chemR23 expression was also demonstrated on endothelial cells [133]. To explain the opposing roles of the chemerin/chemR23 axis, it was alternatively suggested that chemerin exerted its anti-inflammatory properties via chemR23 that was expressed on non-leukocytic (i.e. endothelial or epithelial) cells [121, 136]. In support of this, we observed that the pro-inflammatory activity of chemR23 signaling in the model of DEP-induced acute lung inflammation was restricted to hematopoietic cells that were reported to express chemR23 (i.e. monocytes and DC [284]), while no effects were seen on non-chemR23 expressing cells such as neutrophils. In contrast, in the model of DEP-enhanced allergic airway inflammation, we observed anti-inflammatory activity for both chemR23 expressing and non-expressing cells. Although one could suggest that these anti-inflammatory actions could be mediated through release of chemokines by the endothelium or epithelium, experiments with chimeric mice or conditional chemR23 KO should be performed to definitively resolve this issue.

RvE1 is a second putative ligand of chemR23 [134]. In models of experimental asthma, exogenous administration of RvE1 could both impair the development and promote the resolution of OVA-induced airway inflammation [292-294]. One can therefore speculate that the opposing role of chemR23 signaling in our experiments was due to preferential binding of chemR23 to chemerin (in the model of acute DEP-induced inflammation) or RvE1 (in the model of DEP-enhanced allergic airway inflammation). Although a confounding role for RvE1 cannot be excluded, the decreased RvE1 levels in both the models suggest that the pro- and anti-inflammatory properties of the chemR23 axis in the models is not attributable to RvE1. Indeed, (exogenously administered) chemerin on itself is reported to have both pro- and anti-inflammatory activities [121, 131].

Given the anti-inflammatory role of the chemerin/chemR23 axis in diverse lung disease models, including models of allergic airway inflammation, chemerin or chemR23 agonists are proposed as novel candidate therapeutics for treatment of asthma [121, 290]. On the other hand, blocking chemR23 is also suggested as therapeutic intervention in disease models were the chemR23 axis has pro-inflammatory properties [120]. Our data highlight the complexity of chemerin/chemR23 signaling and the opposing activities depending on the inflammatory conditions. To consider the

chemerin/chemR23 axis as therapeutic target for lung diseases, further research is needed into the mechanisms mediating the pro- versus anti-inflammatory role of chemR23.

CHAPTER 8: IL-33 SIGNALING CONTRIBUTES TO POLLUTANT-INDUCED ALLERGIC AIRWAY INFLAMMATION

IL-33 is an important epithelial-derived cytokine that has been implicated in the pathogenesis of asthma. Recently, an increased IL-33 production was observed in airway epithelial cells derived from severe asthmatics that were exposed to particulate matter. In this study, we aimed to investigate the role of IL-33/ST2 signaling in a murine model of DEPs-enhanced allergic airway inflammation.

De Grove KC*, Provoost S*, Braun H, Teufelberger AR, Krysko O, Beyaert R, Brusselle GG, Joos GF, Maes T. IL-33 signaling contributes to pollutant-induced allergic airway inflammation. Clinical and experimental allergy. **(In revision)**. (*Equal contribution). IF: 5.264, ranking allergy: 4/26.

ABSTRACT

Background: Clinical and experimental studies have identified a crucial role for IL-33 and its receptor ST2 in allergic asthma. Inhalation of traffic-related pollutants, such as diesel exhaust particles (DEP), facilitates the development of asthma and can cause exacerbations of asthma. However, it is unknown whether IL-33/ST2 signaling contributes to the enhancing effects of air pollutants on allergic airway responses.

Objective: We aim to investigate the functional role of IL-33/ST2 signaling in DEP-enhanced allergic airway responses, using an established murine model.

Methods: C57BL/6J mice were exposed to saline, DEP alone, house dust mite (HDM) alone, or combined DEP+HDM. To inhibit IL-33 signaling, recombinant soluble ST2 (r-sST2) was given prophylactically (i.e. during the whole experimental protocol) or therapeutically (i.e. at the end of the experimental protocol). Airway hyperresponsiveness and the airway inflammatory responses were assessed in bronchoalveolar lavage fluid (BALF) and lung.

Results: Combined exposure to DEP+HDM increased IL-33 and ST2 expression in lung, elevated inflammatory responses and bronchial hyperresponsiveness compared to saline, sole DEP or sole HDM exposure. Prophylactic interference with the IL-33/ST2 signaling pathway impaired the DEP-enhanced allergic airway inflammation in the BALF, whereas effects on lung inflammation and airway hyperresponsiveness were minimal. Treatment with r-sST2 at the end of the experimental protocol did not modulate the DEP-enhanced allergic airway responses.

Conclusion: Our data suggest that the IL-33/ST2 pathway contributes to the onset of DEP-enhanced allergic airway inflammation.

INTRODUCTION

Asthma is one of the most common chronic airway diseases and is a serious global health problem [3, 4]. It is generally characterized by a chronic airway inflammation of the conducting airways, causing reversible airway obstruction, excessive mucus production, airway hyperresponsiveness (AHR) and remodeling. Asthma has been defined as a heterogeneous disease, with multiple phenotypes that are distinguished based on the patient's clinical characteristics and inflammatory profile [6]. The best characterized phenotype is allergic asthma, a type 2 mediated immune disorder caused by exposure to common inhaled allergens, such as house dust mite (HDM) [6, 7].

The airway epithelium is continuously exposed to inhaled environmental triggers. It acts not only as a passive barrier but is also actively involved in respiratory defense through pro-inflammatory cytokine production [117]. Interleukin (IL)-33 plays a key role in bridging innate and adaptive immune responses and contributes to the regulation of tissue homeostasis, injury and repair. IL-33 has multiple bioactive forms: a full-length and several more potent cleaved forms. Both forms can bind the ST2 transmembrane receptor that is expressed on various immune cells, including mast cells, basophils, dendritic cells (DC), macrophages, eosinophils, iNKT cells, innate lymphoid cells type 2 (ILC2), $CD4^+$ T-cells and $CD8^+$ T-cells. Besides the transmembrane form, ST2 also exists as a soluble form, sST2, which acts as a decoy receptor to prevent IL-33-mediated cellular activation [139, 140, 295].

Several genome wide association studies have shown that IL-33 and its receptor ST2 are associated with asthma susceptibility [16, 17, 296]. In patients with asthma, elevated IL-33 protein levels were observed in serum, induced-sputum, bronchoalveolar lavage fluid (BALF) and bronchial epithelial cells, which all correlated positively with the severity of the disease [125, 149-151]. Moreover, most murine studies using IL-33 or ST2 knockout (KO) mice, anti-IL-33 or recombinant sST2 (r-sST2) showed a critical role for IL-33/ST2 signaling in allergen-induced airway inflammation [158-160, 163, 172, 235, 297, 298]. Targeting IL-33 could therefore be a new promising therapeutic approach for asthma.

Over the years, substantial epidemiological evidence has linked exposure to traffic-related particulate matter, such as diesel exhaust particles (DEP), with the onset, progression and exacerbation of asthma [77]. Studies with controlled human exposures to DEP + allergen demonstrated augmented allergen-specific responses, including increased airway eosinophilia, type 2 cytokine production and allergen-specific immunoglobulin (Ig) E levels [99, 101]. The underlying mechanisms are however incompletely known. To elucidate these, we and others developed and characterized murine models that mimic DEP-enhanced allergic airway responses [23, 299, 300]. We demonstrated that combined exposure to DEP+HDM was associated with AHR,

increased inflammatory cells in the airways, type 2 cytokine responses, increased immunoglobulin production and mucus metaplasia [299, 300]. Moreover, we observed elevated IL-33 protein levels in lungs of mice that received combined DEP+HDM [300]. *In vitro*, an increased IL-33 production was also observed in PM-exposed epithelial cells derived from severe asthmatics [185]. However, whether IL-33 signaling contributes to the aggravating effect of DEP on allergic airway inflammation remains unknown.

Here, we aim to investigate the role of IL-33/ST2 signaling in DEP-enhanced allergic airway inflammation. For that, r-sST2, which neutralizes IL-33 activity, was administered in our established DEP+HDM mouse model. We demonstrate that interfering with the IL-33/ST2 pathway prophylactically (i.e. during the whole experimental protocol) results in an impaired DEP-enhanced allergic airway inflammation, whereas r-sST2 administration at the end of the exposure protocol does not modulate the DEP-enhanced allergic airway response.

MATERIAL & METHODS

Mice

Female 6-8 weeks old wild-type (WT) C57BL/6J mice (4-8 mice/group) were obtained from the Jackson Laboratory. All *in vivo* manipulations were approved by the Animal Ethical Committee of the Faculty of Medicine and Health Sciences of Ghent University.

Exposure protocol

DEP (SRM 2975) was obtained from the National Institute for Standards and Technology. HDM (Dermatophagoides pterynossinus) was obtained from Greer Laboratories. On day 1, 8 and 15, isoflurane-anesthetized mice were intranasally instilled with saline, 25 µg DEP suspended in saline + 0.05 % tween 80, 1 µg HDM in saline, or the combination of DEP and HDM as described previously [299, 300]. On day 17, the animals were sacrificed by a lethal dose of intraperitoneal pentobarbital **(Fig. 1A)**. Tween 80 (0.05%) was used as a vehicle in all conditions.

For the prophylactic inhibition of IL-33, mice received PBS or 25 µg r-sST2 in PBS intranasally on day 1, 8 and 15. This instillation was performed 8 minutes after the exposure to saline, DEP, HDM or DEP+HDM **(Fig. 1B)** [301]. For the therapeutic approach, mice were treated with PBS or 25 µg r-sST2 in PBS on day 14, 15 and 16 **(Fig. 1C)**.

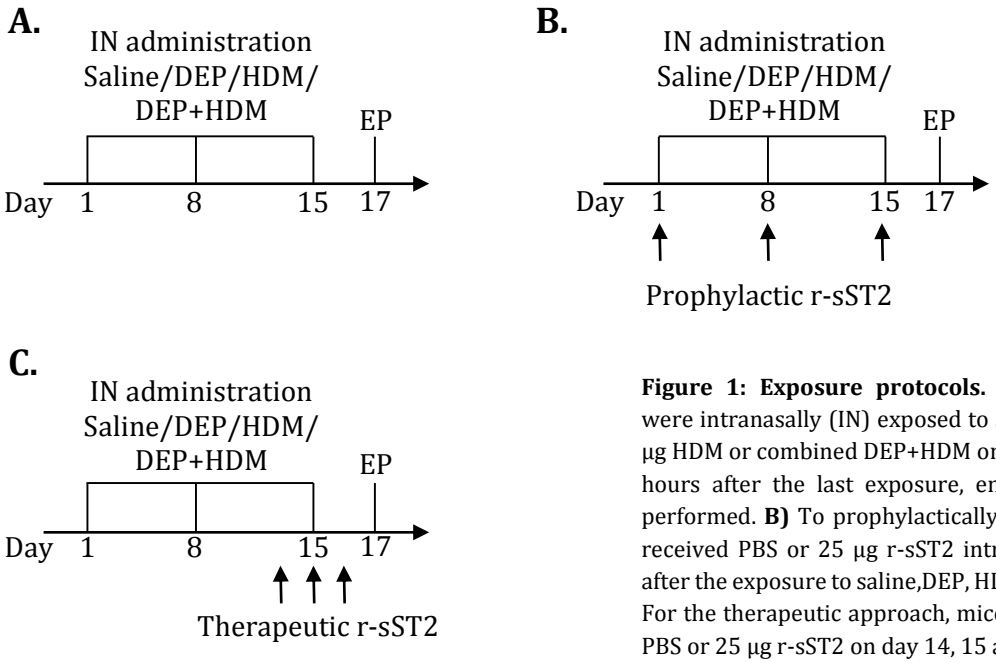


Figure 1: Exposure protocols. A) C57BL/6J mice were intranasally (IN) exposed to saline, 25 µg DEP, 1 µg HDM or combined DEP+HDM on day 1, 8 and 15. 48 hours after the last exposure, endpoints (EP) were performed. **B)** To prophylactically inhibit IL-33, mice received PBS or 25 µg r-sST2 intranasally 8 minutes after the exposure to saline,DEP, HDM or DEP+HDM. **C)** For the therapeutic approach, mice were treated with PBS or 25 µg r-sST2 on day 14, 15 and 16.

BALF

A tracheal cannula was inserted and BALF was recuperated by instillation of 3 x 300 µl 1% HBSS supplemented with 1% BSA and 6 x 500 µl HBSS supplemented with EDTA. The lavage fractions were pooled and total cell counts were measured using a Bürker chamber.

Lung and mediastinal lymph nodes (MLN) single cell suspensions

The pulmonary circulation was rinsed with saline supplemented with EDTA to remove the intravascular pool of cells. Lungs and MLN were minced and incubated for 45 minutes in digestion medium (Roswell Park Memorial Institute medium-1640 supplemented with 5% fetal calf serum, 2 mM L-glutamine, 0.05 mM 2-mercaptomethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mg/ml collagenase type 2, and 0.02 mg/ml DNase I) at 37°C and 5% CO2. Red blood cells were lysed using ammonium chloride buffer. Total cell counts were performed with a Z2 Coulter Counter.

MLN cell culture

MLN single cells were cultured in culture medium alone or supplemented with 3.75 µg/well HDM in round bottom, 96-well plates and incubated in a humidified 37°C incubator with 5% CO2. After 5 days, supernatants were harvested for cytokine measurements.

Flow cytometry and cell sorting

To minimize non-specific binding, BALF cells and lung single cell suspensions were incubated with anti-CD16/CD32 (clone 2.4G2). Cells were further labeled with a combination of anti-mouse fluorochrome-conjugated monoclonal antibodies against: CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), Ly6C (AL-21), Ly6G (1A8), MHCII (2G9), SiglecF (E50-2440), CD3 (145-2C11), CD90.2 (30.H12), CD5 (53-7.3), CD11c (N418), CD25 (PC61.5), CD45R (RA3-6B2), NK1.1 (PK136), TCRβ (H57-597) and ST2 (DJ8). For cytoplasmatic IL-13 (eBio13A) or matched isotype staining, cells were stimulated with ionomycin and phorbol 12-myristate 13-acetate, supplemented with brefeldin A and monensin at 37°C for 4 hours. Data acquisition was performed on a LSR Fortessa cytometer. Cell subsets were analyzed using FlowJo software.

$CD45^+$ and $CD45^-$ lung cells were sorted using an OctoMACS separator and CD45 microbeads following the manufacturer's instructions (Miltenyi).

Histology

The left lung was fixated with 4% PFA and embedded in paraffin. Transversal sections (3 μM) were cut and treated with Ultra V Block. To identify eosinophils and goblet cells, lung sections were stained with congo red or periodic acid-Schiff staining respectively. Quantitative measurements were performed on an Axioimager running Axiovision software

Lung homogenates

The lung was homogenized in T-PER tissue protein extraction reagent containing Halt Protease Inhibitor Cocktail Kit using a TissueRuptor. The homogenates were centrifuged and the middle layer was isolated. Total protein concentration was measured using the Pierce BCA Protein Assay Kit.

Protein measurements

IL-13 levels in BALF and MLN and IL-1β in lung homogenate were measured using commercially available ELISA kits (R&D systems).

HDM-specific IgG1 was determined on serum that was collected by retro-orbital bleeding. Briefly, plates were coated with HDM extract. Serum was added, followed by a horseradish-conjugated polyclonal goat anti-mouse IgG1 Ab. IgG1 values were reported in optical density (ODs).

Western Blotting

Protein extracts of lung tissue homogenates were loaded on a 15% Tris/HCl gel (25 μg/slot), separated by SDS PAGE and blotted onto a nitrocellulose membrane. Murine IL-33 was detected by mouse IL-33 antigen affinity-purified polyclonal goat IgG antibody in combination with the donkey anti-goat horseradish peroxidase (HRP) antibody. As a loading control, rabbit anti–glyceraldehyde-3-phosphate dehydrogenase antibody in combination with donkey anti-rabbit HRP antibody were used. Positive bands were visualized with Immobilon Western

Chemiluminescence HRP substrate and detected by Chemidoc Touch Imaging System. Semi-quantitative analysis of band intensities was performed by measuring the area under the peak of plotted lanes with ImageJ software.

qRT-PCR

RNA from total lung tissue or from CD45+/- sorted lung cells was extracted using the miRNeasy mini kit (Qiagen) and cDNA synthesis (Transcriptor Universal cDNA Master, Roche) was performed following the manufacturer's instructions. IL-33 and ST2 expression was analyzed with TaqMan Gene Expressions assays using a LightCycler 96 system. IL-33 and ST2 expression was normalized based on the expression of the HPRT reference gene.

Airway Hyperresponsiveness

Airway hyperresponsiveness (AHR) in response to increasing doses carbachol (0, 20, 40, 80, 160, 320, 640 μg/kg) was measured 48 hours after the last intranasal instillation of DEP and/or HDM using the forced oscillation technique (Flexivent System). Mice were anaesthetized with a 1/10 dilution pentobarbital and neuromuscular blockade was induced by injecting 1 mg/kg pancuronium bromide intravenously. A "snapshot perturbation" maneuver was imposed to measure the resistance (R) of the whole respiratory system (airways, lung and chest wall).

Statistical analysis

Statistical analysis was performed with SPSS, version 24.0. Non parametric tests (Kruskal-Wallis and Mann-Whitney-U) were used to compare different groups, according to the standard statistical criteria. Values were reported as mean ± SEM. P-values < 0.05 (*) were considered as significant.

RESULTS

Combined DEP+HDM exposure increases IL-33 and ST2 expression in the murine lung

C57BL/6J mice were exposed to saline, DEP alone, HDM alone, or combined DEP+HDM **(Fig. 1A)**. To optimally investigate the enhancing capacity of DEP on HDM-induced allergic airway responses, we have previously optimized doses of both DEP and HDM that elicit limited inflammatory responses on their own [299, 300].

Compared to the three control groups (i.e. saline, DEP alone and HDM alone), combined exposure to DEP+HDM increased the mRNA expression of IL-33 and ST2 in total lung tissue **(Fig. 2A, B)**. To further investigate their origin, we sorted hematopoietic ($CD45^+$) versus non-hematopoietic ($CD45^-$) cells from lung tissue. IL-33 mRNA was predominantly expressed in $CD45^-$ non-hematopoietic cells, while it was barely detectable in $CD45^+$ hematopoietic cells **(Fig. 2C)**. Contrary to this, ST2 mRNA was preferentially expressed in the $CD45^+$ cell population, whereas it was minimally expressed in the $CD45^-$ cell fraction **(Fig. 2D)**. Similar to the data from total lung tissue, combined exposure to DEP+HDM increased expression of IL-33 in the sorted $CD45^-$ cells and ST2 mRNA in the sorted $CD45^+$ cells, when compared with the three control groups **(Fig. 2C and D)**.

We previously showed that IL-33 protein levels in lung homogenates increased upon combined DEP+HDM-exposure compared to saline, DEP and HDM exposure [300]. To identify which protein form of IL-33 was increased, we performed western blot analysis. Both full-length IL-33 (~30kDa) and cleaved IL-33 (~18kDa) were significantly increased in WT mice that received combined DEP+HDM in comparison with the three control groups **(Fig.2E-G)**.

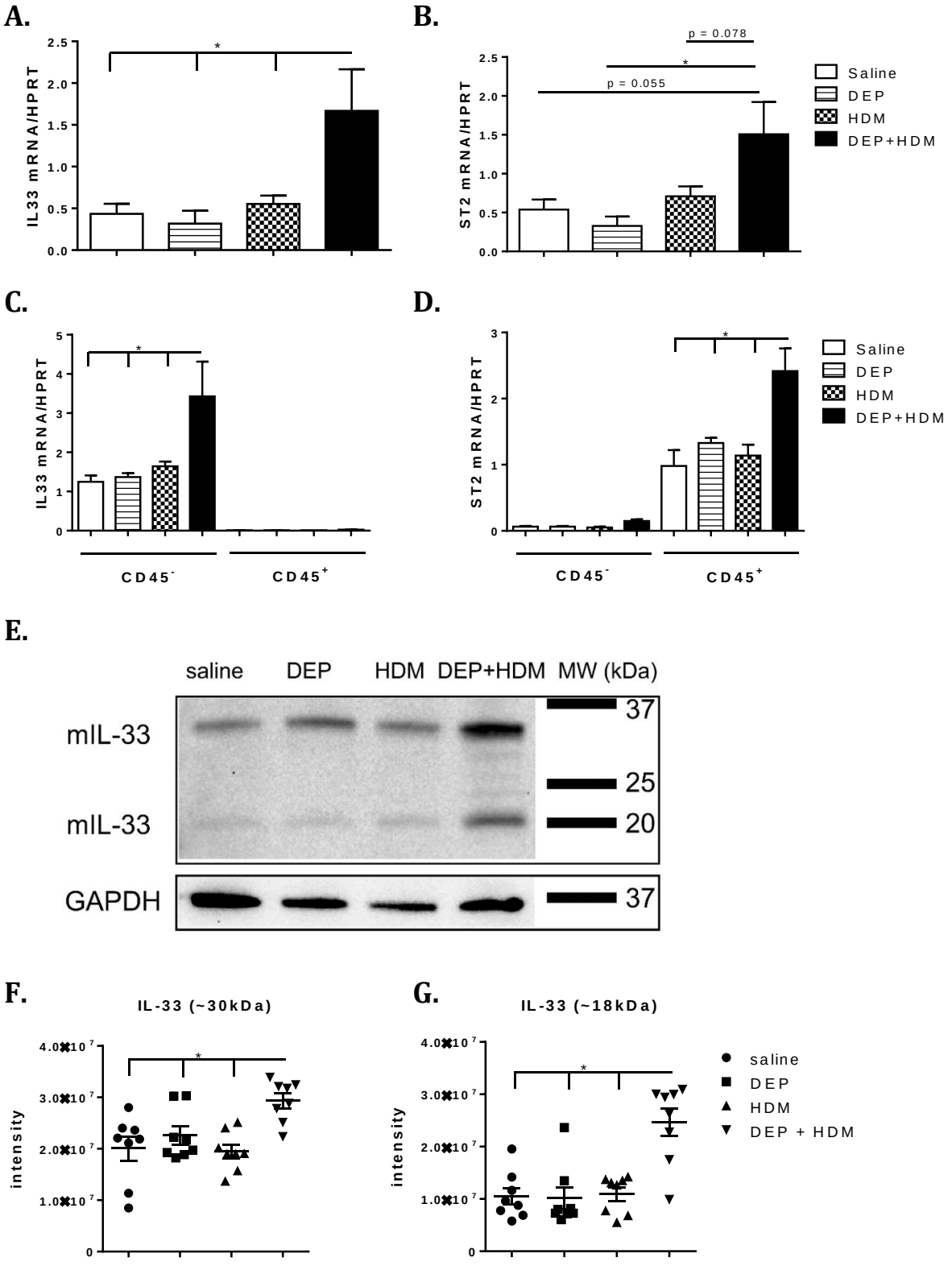


Figure 2: Combined DEP+HDM exposure increases IL-33 and ST2 expression in the murine lung. C57BL/6J WT mice were intranasally exposed to saline (white bars), 25 µg DEP (striped bar), 1 µg HDM (checked bar) or combined DEP+HDM (black bar). **A-B,** IL-33 **(A)** and ST2 **(B)** mRNA expression in total lung tissue was determined using qRT-PCR. **C-D,** IL-33 **(C)** and ST2 **(D)** mRNA expression in sorted non-hematopoietic ($CD45^-$) and hematopoietic ($CD45^+$) lung cells was determined with qRT-PCR. **E-G,** Full-length and cleaved murine IL-33 in lung homogenates were examined by western blot and relative band-intensities were measured. Results are expressed as mean ± SEM. n = 4-8 mice per group. * $P < 0.05$.

Prophylactic administration of recombinant soluble ST2 attenuates DEP-enhanced allergic airway inflammation in BALF

To evaluate the functional role of the IL-33/ST2 axis in the model of DEP-enhanced allergic airway inflammation, we inhibited IL-33/ST2 signaling by administering r-sST2 or PBS control prophylactically (i.e. from the start of the exposure protocol) to C57BL/6J mice **(Fig. 1B)**.

Combined exposure to DEP+HDM in PBS-treated mice vastly increased the numbers of DC, neutrophils, eosinophils, ILC2 and $CD4^+$ T cells in the BALF **(Fig. 3A-E)**, whereas exposure to sole DEP or sole HDM elicited minimal inflammation. Moreover, elevated IL-13 protein levels and increased numbers of IL-13^+ ILC2 and IL-13^+ $CD4^+$ T cells were observed in the BALF of combined DEP+HDM-exposed mice, when compared to saline-, DEP- or HDM-exposed mice **(Fig. 3F-H)**. Concomitant exposure to DEP+HDM modestly increased the IL-1β levels compared with the controls **(Fig. 3I)**.

Upon prophylactic administration of r-sST2, the DEP-enhanced allergic airway inflammation was significantly reduced. r-sST2-treated mice had reduced numbers of DC, neutrophils, eosinophils, ILC2 and $CD4^+$ T cells in BALF on exposure to combined DEP+HDM, when compared with the PBS-treated mice that received DEP+ HDM **(Fig. 3A-E)**. In addition, IL-13 protein levels as well as IL-13^+ ILC2 and IL-13^+ $CD4^+$ T-cells in BALF were decreased in DEP+HDM-exposed mice that were treated with r-sST2, in comparison with the PBS-treated DEP+HDM group **(Fig. 3F-H)**. Moreover, administration of r-sST2 lowered the IL-1β levels in the DEP+HDM-exposed mice compared with the PBS-treated DEP+HDM controls **(Fig. 3I)**. It has to be acknowledged however that r-sST2 treatment did not completely abolish the allergic airway inflammation, since combined DEP+HDM-exposed mice treated with r-sST2 still had higher inflammatory responses in the BALF when compared to saline-, DEP- or HDM-exposed mice treated with r-sST2 **(Fig. 3A-H)**. Of interest, in mice that were exposed to sole HDM, prophylactic treatment with r-sST2 also reduced the number of ILC2 and IL-13^+ ILC2 in the BALF when compared to the PBS-treated HDM group **(Fig. 3D, G)**.

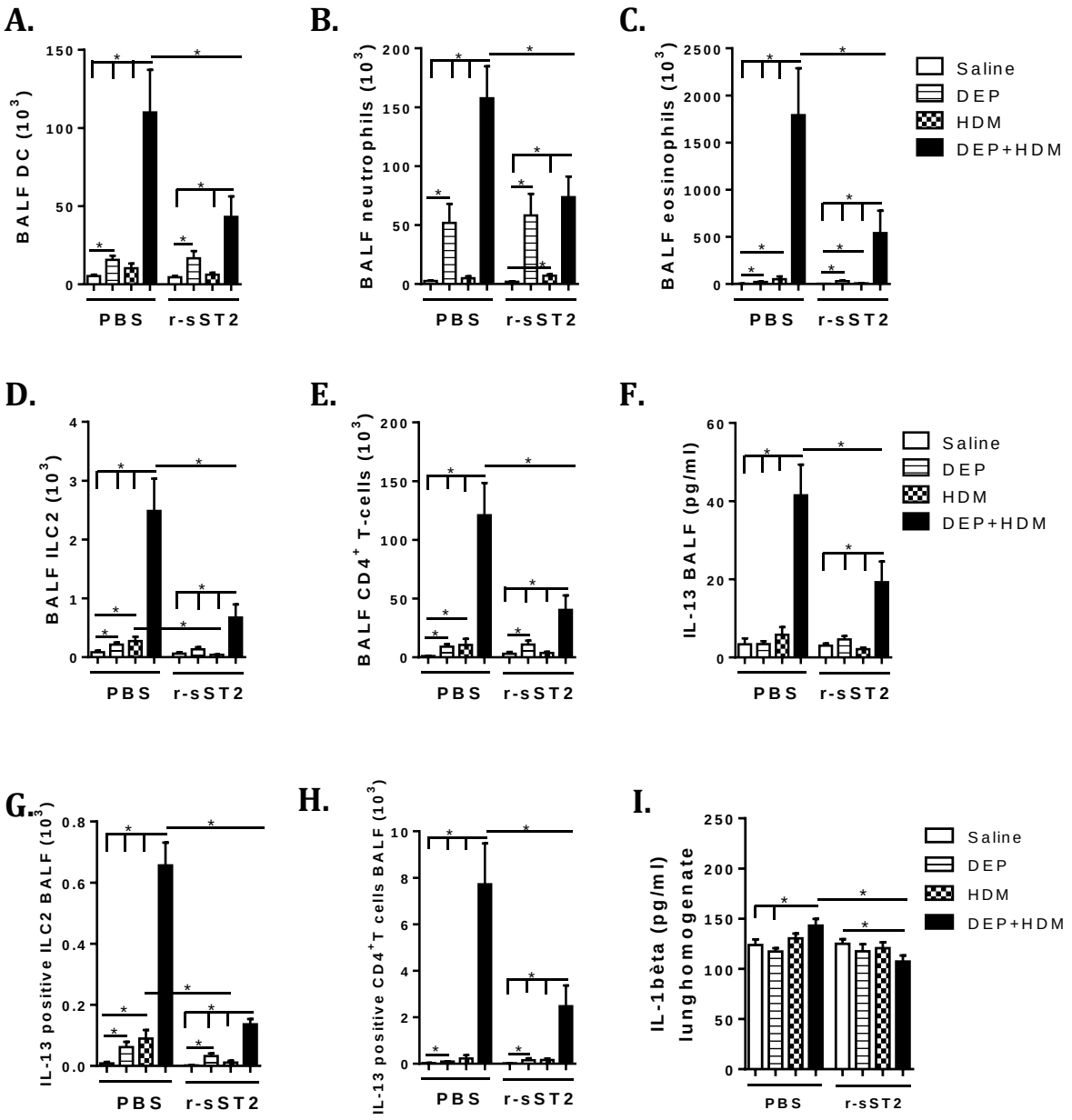


Figure 3: Prophylactic administration of recombinant soluble ST2 attenuates DEP-enhanced allergic airway inflammation in BALF. C57BL/6J mice were intranasally exposed to saline (white bars), 25 µg DEP (striped bars), 1 µg HDM (checked bars) or combined DEP+HDM (black bars). 8 minutes after each exposure, the mice were treated with PBS or 25 µg r-sST2. **A-E,** DC ($CD45^+$, $CD11c^{high}$, and $MHCII^+$) **(A)**, neutrophils ($CD45^+$, $CD11c^-$, $CD11b^+$, $Ly6G^+$, and $Ly6C^+$) **(B)**, eosinophils ($CD45^+$, $CD11c^-$, $CD11b^+$, and $Siglec\text{-}F^+$) **(C)**, ILC2 (CD45+, Lin^- ($CD3^-$, $CD5^-$, $NK1.1^-$, $TCR\beta^-$, $CD11c^-$, $CD11b^-$ and $CD45R^-$), $CD25^+$, $CD90^+$, and $ST2^+$) **(D)** and $CD4^+$ T cells ($CD45^+$, $CD3^+$, $CD4^+$, and $CD8^-$) **(E)** in BALF were determined by flow cytometry. **F,** IL-13 protein levels in BALF were determined by ELISA. **G-H,** BALF cells were stimulated for 4 hours with PMA/ionomycin + protein transport inhibitors, intracellularly labeled and analyzed using flow cytometry. Number of IL-13 expressing ILC2 ($CD45^+$, Lin^-, $CD25^+$, and $CD90^+$) **(G)** or $CD4^+$ T cells ($CD45^+$, $CD3^+$, and $CD4^+$) **(H)**. **I,** IL-1β levels in lung homogenate were determined by ELISA. Results are expressed as mean ± SEM. n = 8 mice per group. * $P < 0.05$.

Prophylactic r-sST2 administration does not prevent tissue inflammation, type 2 sensitization and airway hyperresponsiveness upon combined DEP+HDM exposure

Combined DEP+HDM-exposed mice that were treated with PBS showed a significant higher amount of peribronchial eosinophils and goblet cell metaplasia in lung tissue, compared to saline-, sole DEP- or sole HDM-exposed mice **(Fig. 4A, B)**. Moreover, concomitant DEP+HDM exposure in PBS-treated animals was associated with increased IL-13 production in the mediastinal lymph nodes (MLN) and elevated serum HDM-specific IgG1 levels **(Fig. 4C-D)**.

Prophylactic interference with the IL-33/ST2 pathway had no significant effect on peribronchial eosinophilic inflammation, goblet cell metaplasia, IL-13 levels in MLN and HDM-specific IgG1 levels in serum since similar levels were observed in DEP+HDM-exposed mice that were treated with r-sST2 in comparison with DEP+HDM group that was treated with PBS **(Fig. 4A-D)**. Of note, although r-sST2 lowered the amount of peribronchial eosinophils and goblet cells in HDM-exposed mice compared to their PBS controls, this was not statistically significant (**Fig. 4A, B)**.

Interestingly, mice that were concomitantly exposed to DEP+HDM showed airway hyperresponsiveness (AHR) compared to sole HDM-exposed mice. Prophylactically administering r-sST2 to DEP+HDM-exposed mice led to a slight reduction – although not significantly – in AHR **(Fig. 4E)**.

Therapeutic r-sST2 administration does not modulate the DEP-enhanced allergic airway inflammation

To investigate the therapeutic potential of r-sST2 in DEP-enhanced allergic airway inflammation, we administered r-sST2 only at the end of the exposure protocol **(Fig. 1C)**.

Combined DEP+HDM-exposed mice that were therapeutically treated with r-sST2 had a similar increase in BALF DC, neutrophils, eosinophils, ILC2 and $CD4^+$ T cells, compared with the PBS-treated DEP+HDM group **(Fig. 5A-E)**. Moreover, the increased IL-13 protein levels and total number of $IL\text{-}13^+$ ILC2 and $CD4^+$ T cells in the BALF did not differ between DEP+HDM-exposed mice that were treated with PBS or r-sST2 **(Fig. 5F-H)**. A similar increase in IL-1β levels was observed in DEP+HDM-exposed mice that were treated with PBS or r-sST2 **(Fig. 5I)**.

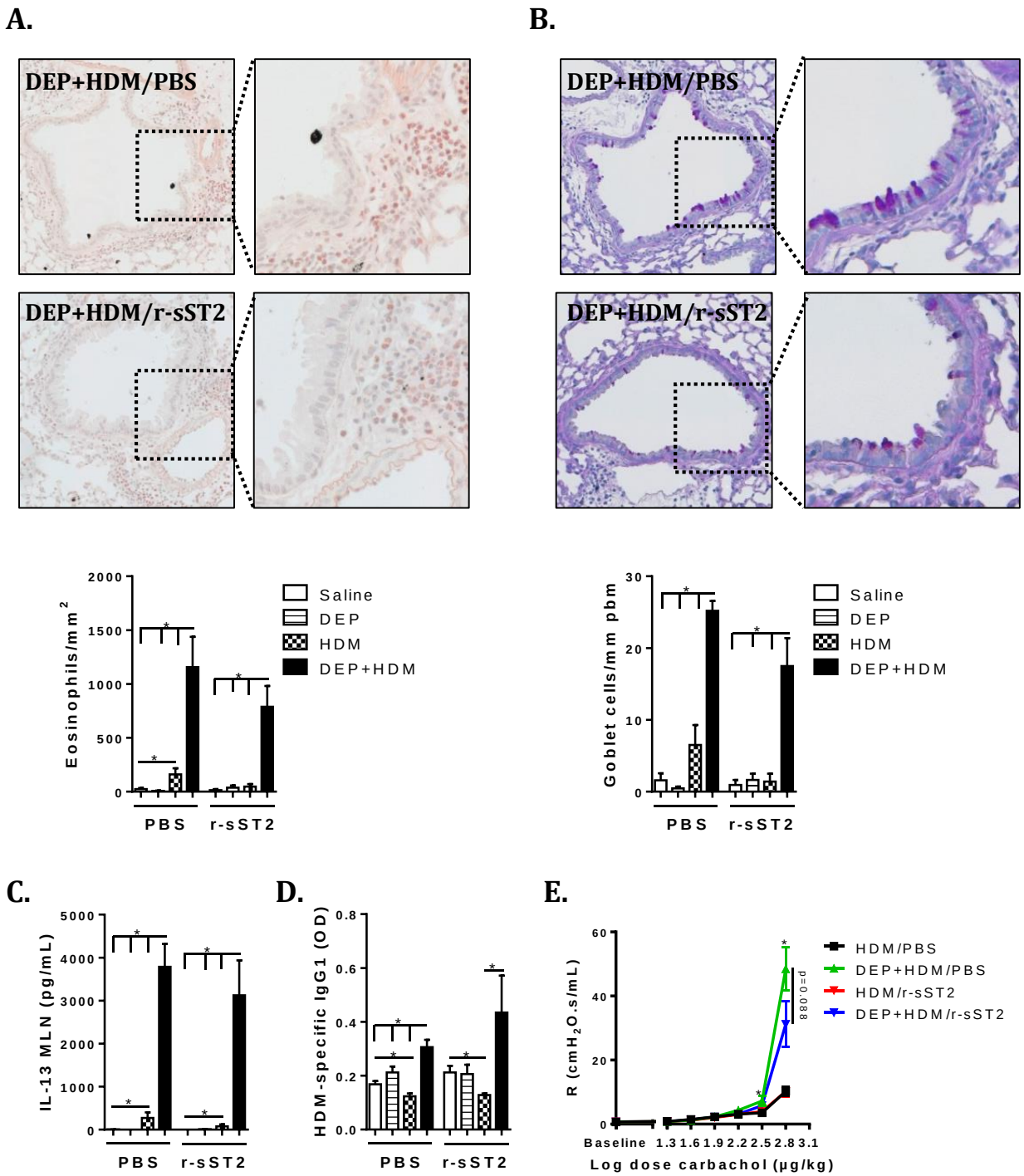


Figure 4: Prophylactic r-sST2 administration does not prevent tissue inflammation, type 2 sensitization and airway hyperresponsiveness upon combined DEP+HDM exposure. C57BL/6J mice were intranasally exposed to saline (white bars), 25 µg DEP (striped bars), 1 µg HDM (checked bars) or combined DEP+HDM (black bars). 8 minutes after each exposure, the mice were treated with PBS or 25 µg r-sST2. **A-B,** Representative photomicrographs and quantification of congo red-stained peribronchial eosinophils **(A)** or periodic acid-Schiff-stained mucus-producing goblet cells **(B)**. **C-D,** IL-13 levels in the supernatants of MLN restimulated with HDM **(C)** and HDM-specific IgG1 levels in serum **(D)** were determined by ELISA. **E,** Airway resistance (R) of HDM-exposed mice treated with PBS (black line), combined DEP+HDM-exposed mice treated with PBS (green line), HDM-exposed mice treated with r-sST2 (red line) and DEP+HDM-exposed mice treated with r-sST2 (blue line) was measured in response to increasing doses of carbachol. Results are expressed as mean ± SEM. n = 5-8 mice per group. * $P < 0.05$.

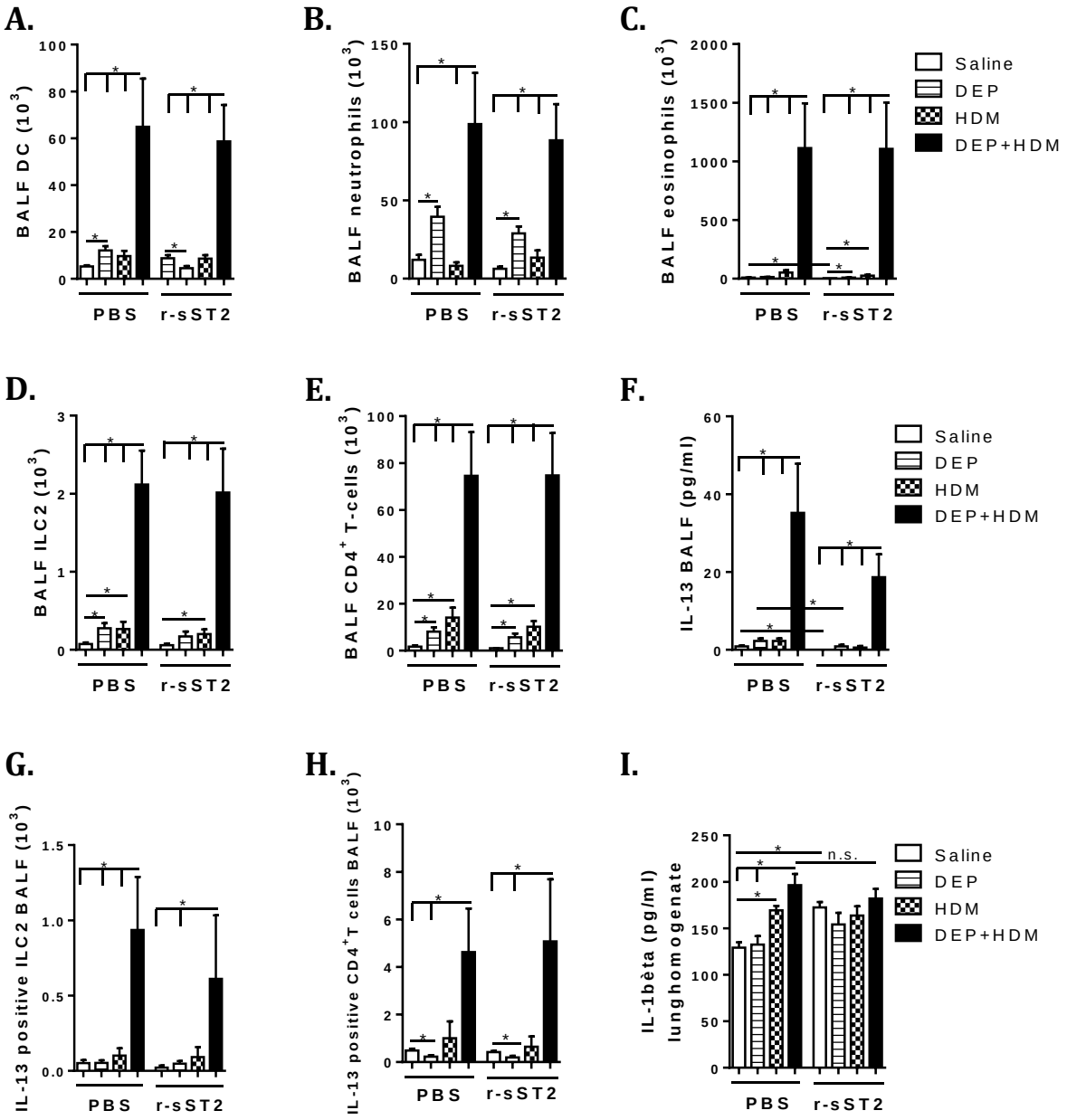


Figure 5: Therapeutic r-sST2 administration does not modulate the DEP-enhanced allergic airway inflammation in the BALF. C57BL/6J mice were intranasally exposed to saline (white bars), 25 µg DEP (striped bars), 1 µg HDM (checked bars) or combined DEP+HDM (black bars). On day 14, 15 and 16, mice were treated with PBS or 25 µg r-sST2. **A-F,** DC ($CD45^+$, $CD11c^{high}$, and $MHCII^+$) **(A)**, neutrophils ($CD45^+$, $CD11c^-$, $CD11b^+$, $Ly6G^+$, and $Ly6C^+$) **(B)**, eosinophils ($CD45^+$, $CD11c^-$, $CD11b^+$, and $Siglec\text{-}F^+$) **(C)**, ILC2 ($CD45^+$, Lin^- ($CD3^-$, $CD5^-$, $NK1.1^-$, $TCR\beta^-$, $CD11c^-$, $CD11b^-$ and $CD45R^-$), $CD25^+$, $CD90^+$, and $ST2^+$) **(D)** and $CD4^+$ T cells ($CD45^+$, $CD3^+$, $CD4^+$, and $CD8^-$) **(E)** in BALF were determined by flow cytometry. **F,** IL-13 protein levels in BALF were determined by ELISA. **G-H,** BALF cells were stimulated for 4 hours with PMA/ionomycin + protein transport inhibitors, intracellularly labeled and analyzed using flow cytometry. Number of IL-13 expressing ILC2 ($CD45^+$, Lin^-, $CD25^+$, and $CD90^+$) **(G)** or $CD4^+$ T cells ($CD45^+$, $CD3^+$, and $CD4^+$) **(H)**. **I,** IL-1β levels in lung homogenate were determined by ELISA. Results are expressed as mean ± SEM. n = 4-8 mice per group. * $P < 0.05$.

Histological analyses further revealed a comparable peribronchial eosinophilia and goblet cell metaplasia in the lungs of DEP+HDM-exposed mice that were treated with PBS or r-sST2. Despite the fact that r-sST2 lowered the amount of peribronchial eosinophils and goblet cells in HDM-exposed mice compared to their PBS controls, this could not be statistically confirmed **(Fig. 6A, B)**.

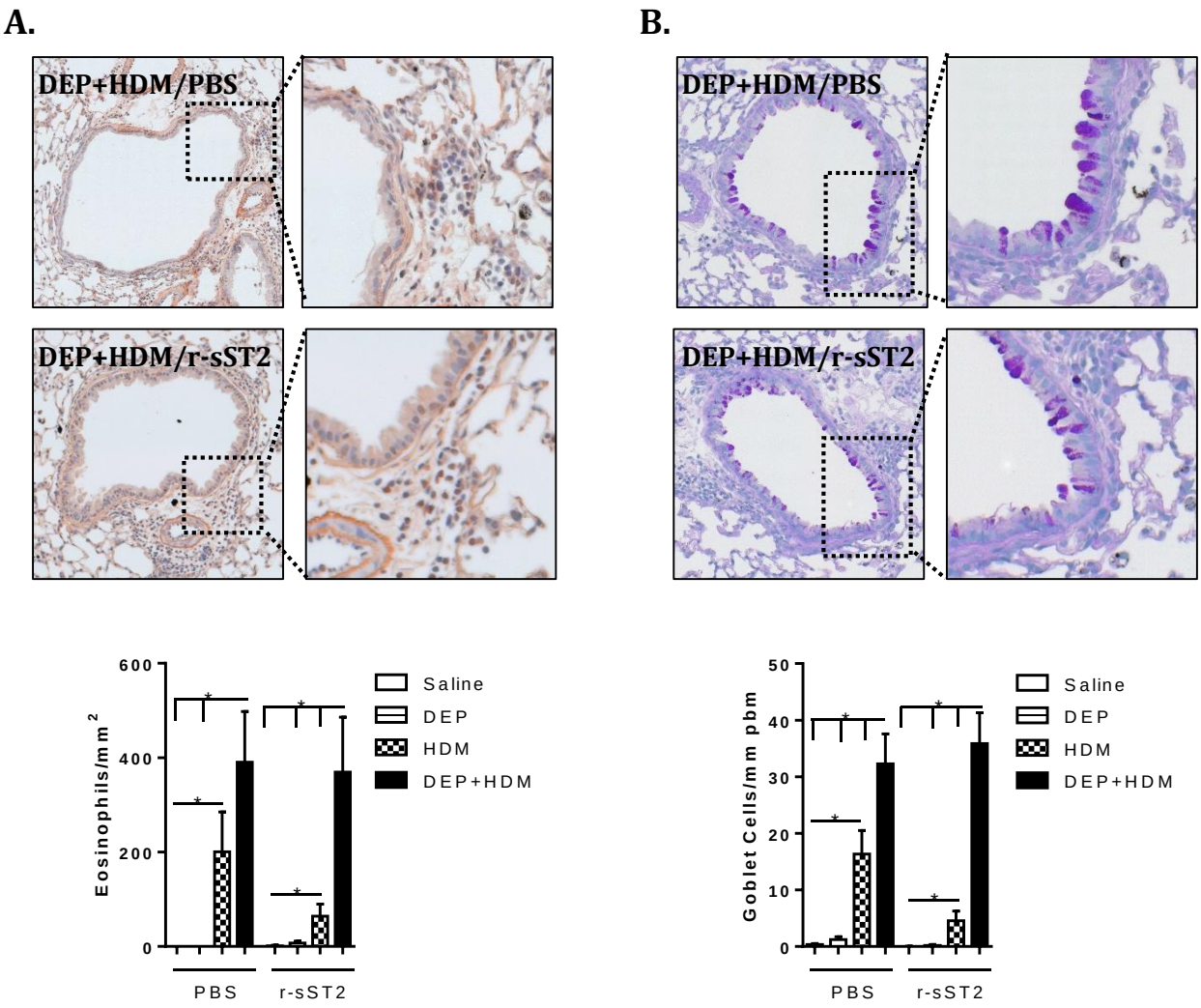


Figure 6: Therapeutic r-sST2 administration does not modulate the DEP-enhanced allergic lung inflammation. C57BL/6J mice were intranasally exposed to saline (white bars), 25 µg DEP (striped bars), 1 µg HDM (checked bars) or combined DEP+HDM (black bars). On day 14, 15 and 16, mice were treated with PBS or 25 µg r-sST2. **A-B,** Representative photomicrographs and quantification of congo red-stained peribronchial eosinophils **(A)** periodic acid-Schiff-stained mucus-producing goblet cells **(B)**. Results are expressed as mean ± SEM. n = 7-8 mice per group. * P < 0.05.

DISCUSSION

In this paper, we focused on the functional role of IL-33/ST2 signaling in the aggravating effect of DEP on HDM-induced airway responses. We demonstrate that a prophylactic neutralization of IL-33 activity with r-sST2 attenuates allergic airway inflammation upon combined DEP+HDM exposure. In contrast, in a therapeutic setting, in which r-sST2 is given only at the end of the exposure protocol, the DEP-enhanced allergic airway response is not affected.

IL-33 is an important epithelial-derived cytokine that has been implicated in the pathogenesis of asthma. Indeed, increased IL-33 expression was already demonstrated in asthmatic patients which correlated with asthma severity [125]. Also in murine models of asthma, a rapid IL-33 increase in BALF and lung tissue was observed upon allergen exposure [159, 161, 297]. Although in our model no increase in lung IL-33 levels was observed in response to suboptimal HDM doses, concomitant DEP+HDM exposure vastly increased the pulmonary IL-33 mRNA and protein levels.. In accordance, *in vitro* exposure of human bronchial epithelial cells from severe asthmatics to PM resulted in increased IL-33 production [185]. Although under inflammatory conditions IL-33 can be induced in immune cells [140], we identified non-hematopoietic cells as the major IL-33 producers in the model of DEP-enhanced allergic airway inflammation, whereas ST2 was predominantly present on the hematopoietic cells. Moreover, although this should be further examined, the increase in cleaved IL-33 that was seen upon combined exposure to DEP+HDM could have important implications, as cleaved IL-33 can be up to 30-fold more potent than the full-length IL-33 form [139, 295].

To assess the functional role of the IL-33/ST2 pathway in DEP-enhanced allergic airway responses, r-sST2 was intranasally administered from the start of the exposure protocol. We demonstrated that this prophylactic administration of r-sST2 in DEP+HDM-exposed mice decreased numbers of eosinophils, T_H2 cells and ILC2 and IL-13 levels in the BALF. In contrast, r-sST2 had no significant effect on the DEP+HDM-induced responses in lung tissue, HDM-specific IgG1 levels and AHR. Considering that only a local response towards r-sST2 was observed, whereas the effects in lung tissue and draining LN were limited, it could be that the half-life of r-sST2 was too short or dosing was suboptimal due to the intranasal administration route. In a model of SplD-induced asthma however, intratracheal administration of r-sST2 also minimally affected the inflammatory responses in the lung tissue [301]. Furthermore, an i.p. injection of r-sST2 at the time of HDM sensitization showed also no apparent differences in type 2 immune response in the lymph nodes and amount of goblet cells [161]. Finally, it should be mentioned that – at least in murine models of asthma – discrepant results have been reported concerning the functional role of IL-33 signaling. Although a reduction of the HDM- or OVA-induced allergic

airway inflammation and AHR was shown in most studies with IL-33 KO mice, ST2 KO mice or mice that were treated with anti-IL-33 or r-sST2 [159, 160, 163, 298, 302], some studies found no differences [165, 166]. Of note, with exception of the total number and cytokine-producing ILC2 in the BALF, also in our model, r-sST2 did not significantly affect allergic airway responses towards suboptimal doses of sole HDM.

As it was previously demonstrated that anti-IL-33 treatment could decrease a PM-induced asthma exacerbation in mice [186], we were interested if r-sST2 could be therapeutically beneficial during the aggravating effects of DEP on allergic airway inflammation. When r-sST2 was administered only at the end of the exposure protocol, no differences in airway immune responses could be observed, suggesting that signaling via the IL-33/ST2 pathway is more important during the onset of DEP-enhanced allergic airway inflammation. Our observations are in line with data from mouse models of HDM-induced asthma, in which it was shown that administration of r-sST2 during the challenge phase had no effect on airway eosinophilia [161], whereas IL-33 neutralization at the time of sensitization attenuated the HDM-induced eosinophil and lymphocytic inflammation in the BALF [161, 162]. We can however not exclude that the lack of efficacy in this therapeutic setting could be due to the half-life of r-sST2 or intranasal application route . In this regard, other approaches could give additional insights concerning the potential therapeutic effect of IL-33 blockage.

The mechanisms underlying the discrepant results of IL-33 during the prophylactic and therapeutic setting should be further investigated. As decreased IL-1β levels were observed in DEP+HDM-exposed mice that were prophylactically treated with r-sST2, but not therapeutically, this could be a potential mechanism of the observed discrepancy in inflammatory airway responses since IL-1β was previously found to be involved in type 2 promoting responses [303, 304].

At the moment, human studies with regard to anti-IL-33 are ongoing [37, 168] and still have to prove their beneficial effect in asthmatic patients. Moreover, it would be interesting to examine the functional role of other epithelial type 2 promoting cytokines, i.e. IL-25 and TSLP, during DEP-enhanced allergic airway inflammation, as it was suggested that combinatorial blockage of these cytokine could be necessary to effectively inhibit lung inflammation [272].

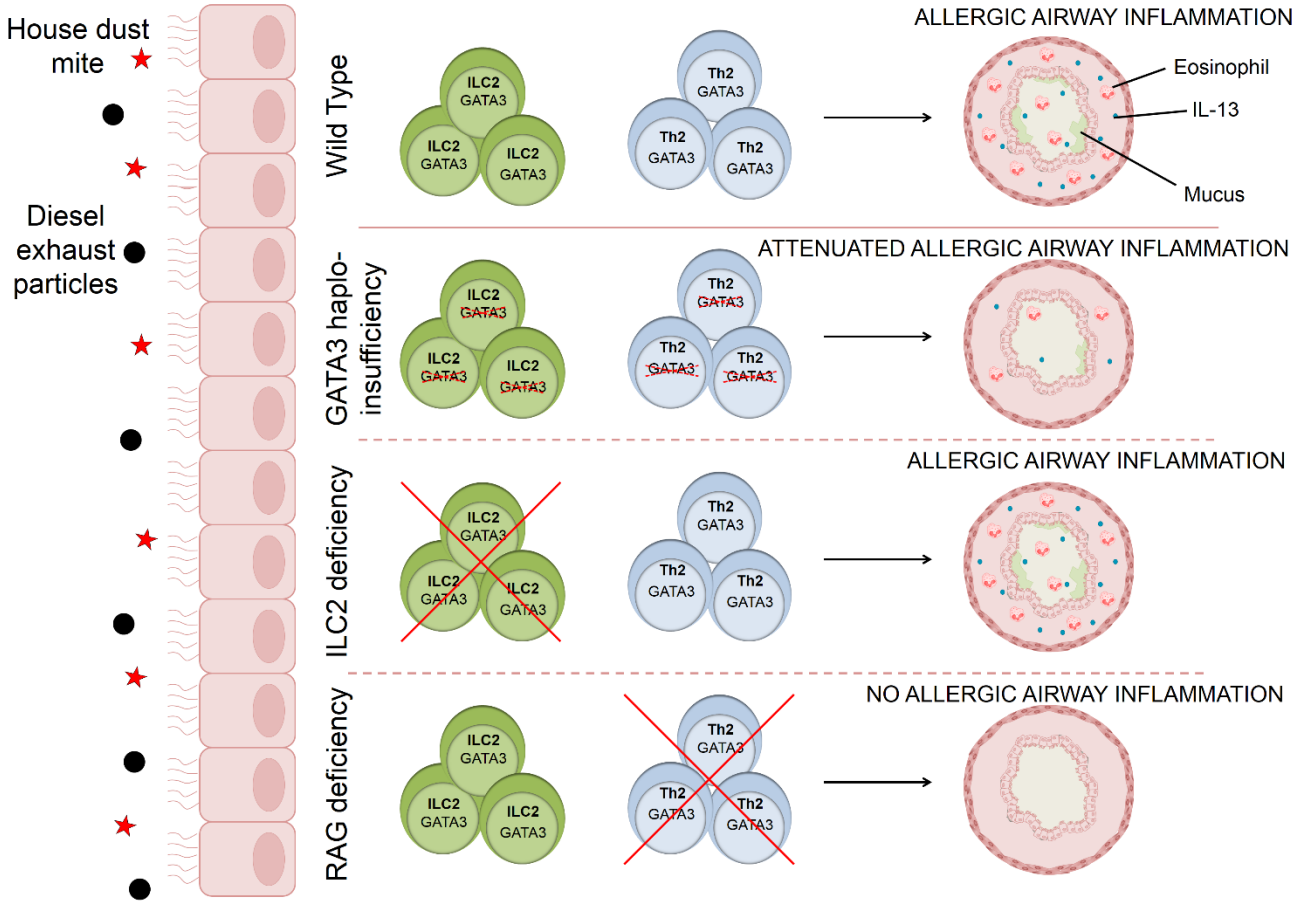
In conclusion, our preclinical data suggest that signaling through the IL-33/ST2 pathway has a functional role during the onset of DEP-enhanced allergic airway inflammation, whereas interference with IL-33 signaling in established DEP+HDM-induced inflammation is ineffective.

CHAPTER 9: DYSREGULATION OF TYPE 2 INNATE LYMPHOID CELLS AND T_H2 CELLS IMPAIRS POLLUTANT-INDUCED ALLERGIC AIRWAY RESPONSES

Although allergic asthma is typically considered as a T_H2-mediated disease, an important contribution of ILC2s in allergic airway inflammation has been identified. Besides allergen exposure, it has been well accepted that exposure to pollutants can induce and exacerbate the allergic inflammatory responses. The underlying mechanisms remain however largely unknown. In this study, we aimed to investigate the role of both ILC2s and T_H2 cells during DEP-enhanced allergic airway inflammation.

De Grove KC*, Provoost S*, Hendriks RW, McKenzie ANJ, Seys JM, Kumar S, Maes T, Brusselle GG, Joos GF. Dysregulation of type 2 innate lymphoid cells and Th2 cells impairs pollutant-induced allergic airway responses. Journal of Allergy and Clinical Immunology. 2017; 139(1):246-257. (*Equal contribution).

GRAPHICAL ABSTRACT



ABSTRACT

Background: Whereas the prominent role of T helper 2 (T_H2) cells in type 2 immune responses is well established, the newly identified type 2 innate lymphoid cells (ILC2) can also contribute to the orchestration of allergic responses. Several experimental and epidemiological studies have provided evidence that allergen-induced airway responses can be further enhanced upon exposure to environmental pollutants, such as diesel exhaust particles (DEP). However, the components and pathways responsible remain incompletely known.

Objective: To investigate the relative contribution of ILC2 and adaptive T_H2 cell responses in a murine model of DEP-enhanced allergic airway inflammation.

Methods: Wild-type (WT), $Gata3^{+/nlslacZ}$ (Gata-3 haploinsufficient), $ROR\alpha^{fl/flIL7RCre}$ (ILC2-deficient) and $Rag2^{-/-}$ mice were challenged with saline, DEP or house dust mite (HDM), or combined DEP+HDM. Airway hyperresponsiveness as well as inflammation and intracellular cytokine expression in ILC2 and T_H2 cells in the bronchoalveolar lavage fluid and lung tissue were assessed.

Results: Concomitant DEP+HDM exposure significantly enhanced allergic airway inflammation, characterized by increased airway eosinophilia, goblet cell metaplasia, accumulation of ILC2 and T_H2 cells, type 2 cytokine production and airway hyperresponsiveness, compared to sole DEP or HDM. Reduced Gata-3 expression decreased the number of functional ILC2 and T_H2 cells in DEP+HDM exposed mice, resulting in an impaired DEP-enhanced allergic airway inflammation. Interestingly, whereas the DEP-enhanced allergic inflammation was marginally reduced in ILC2-deficient mice that received combined DEP+HDM, it was abolished in DEP+HDM-exposed $Rag2^{-/-}$ mice.

Conclusion: These data indicate that dysregulation of ILC2 and T_H2 cells attenuates DEP-enhanced allergic airway inflammation. In addition, a crucial role for the adaptive immune system was shown upon concomitant DEP+HDM exposure.

INTRODUCTION

Asthma is a chronic disorder of the conducting airways that is associated with a reversible airway obstruction, chronic airway inflammation, airway remodeling, and airway hyperresponsiveness [10]. It is a heterogeneous disease where multiple phenotypes can be distinguished based on clinical characteristics and inflammatory profile. Asthma that originates during childhood (early-onset asthma) mostly has an atopic component [6, 270] and is typically considered as mainly a T helper 2 (T_H2) driven disease [305].

In addition to the adaptive immune system, the airway epithelium has gained great importance during the initiation and maintenance of the allergic and asthmatic cascade. In particular, it has been shown that upon allergen exposure, several epithelial cytokines – such as interleukin (IL)-25, IL-33 and thymic stromal lymphopoietin (TSLP) – are involved in the pathogenesis of asthma [102, 306]. Moreover, several genes discovered in genome wide association studies (i.e. IL-33, IL-1RL1, TSLP) support a key role for these cytokines [16, 17]. On the one hand, these epithelial-derived cytokines have the capability to activate the adaptive immune system by stimulating T_H2 polarizing dendritic cells (DC) [102]. On the other hand, the recently identified type 2 innate lymphoid cells (ILC2) also become activated by these cytokines [307-309]. Analogous with the T_H2 cells, ILC2 require the transcription factor Gata-3 and are a potent source of the type 2 cytokines IL-5 and IL-13, which are able to induce lung eosinophilia and mucus hypersecretion [170, 199, 234, 235, 310]. Studies in $Rag^{-/-}$ mice have shown that these ILC2 are crucial players in allergic airway responses [311]. Even in the absence of the adaptive immune system, ILC2 were able to mediate eosinophilia, goblet cell metaplasia, type 2 cytokine production and airway hyperresponsiveness (AHR) [241, 242, 312]. In addition, mice that were ILC2-deficient due to the targeting of the transcription factor RORα had decreased type 2 immune responses [170, 245, 249]. Interestingly, it was reported that ILC2 and T cells interact with each other and that this crosstalk could contribute to the maintenance, proliferation and activation of both ILC2 and T_H2 cells [248, 249, 252].

Besides allergen exposure, it has become well accepted that traffic-related particulate matter, such as diesel exhaust particles (DEP), also contributes to the development and exacerbation of asthma [23, 60, 313]. For instance, epidemiological studies reported a correlation between high DEP levels and the frequency of symptomatic episodes in allergic children [314]. In addition, combined allergen + DEP administration during controlled human exposure studies resulted in increased allergen-specific immunoglobulins and type 2 cytokine responses [99]. Furthermore, concomitant DEP + house dust mite (HDM) exposure in murine models enhanced eosinophilia, immunoglobulin production, AHR and remodelling [96]. However, the mechanisms underlying

the enhanced effects of DEP on allergen-induced airway inflammation remain largely unknown. Several studies suggested that the airway epithelium could be an important player since particulate matter was also able to stimulate the release of several epithelial-derived cytokines, such as TSLP and IL-33, which can lead to enhanced DC maturation and T_H2 responses [94, 95, 122, 186]. However, whether this also activates ILC2 is unknown.

In this paper, we investigate the relative contribution of ILC2 and the adaptive immune system in the enhancing effects of DEP on allergen-induced airway inflammation. We show in a murine model that concomitant exposure to a clinically relevant allergen (i.e. HDM) and DEP enhances several allergic airway responses, including airway eosinophilia, goblet cell metaplasia, increased ILC2 and T_H2 cells, type 2 cytokine production and AHR. Since Gata-3 is an important transcription factor during the development and function of both ILC2 and T_H2 cells [315], we used haploinsufficient $Gata3^{+/nlslacZ}$ mice – which have a reduced expression of Gata-3 [316] – to demonstrate that the enhancing effects of DEP on allergic airway inflammation depend on Gata-3 mediated regulation of ILC2 and T_H2 cells. Moreover, to examine the specific contribution of ILC2 in the model of DEP-enhanced allergic airway inflammation, we used a conditionally targeted $ROR\alpha^{fl/fl}$ mouse that, when intercrossed with IL-7 receptor (IL7R)-Cre mice, yields an ILC2-deficient mouse strain in which other lineages are unaffected [249]. Finally, to investigate the functional role of the adaptive immune system in this model, we used $RAG2^{-/-}$ mice which lack mature T and B cells [317]. We demonstrated that ILC2 marginally contributed to DEP-enhanced allergic airway responses, whereas the adaptive immune system appeared critical to orchestrate the enhanced effect of DEP on allergic airway inflammation and AHR.

MATERIAL & METHODS

Mice

Female C57BL/6 mice (6-8 weeks old) were obtained from the Jackson Laboratory. C57BL/6 Gata3$^{+/nlslacZ}$ and their WT littermates were a kind gift of Dr. R. Hendriks (Department of pulmonary medicine, Erasmus MC, Rotterdam, The Netherlands) [316] and bred in our animal facility of Ghent University. RORα$^{fl/fl}$IL7R^{Cre} mice and RORα$^{fl/fl}$IL7R$^{+/+}$ controls were on a C57BL/6 background [249]. C57BL/6 Rag2$^{-/-}$ mice [317] and their wild-type (WT) controls were purchased from Taconic. All *in vivo* manipulations were approved by the Animal Ethical Committee of the Faculty of Medicine and Health Sciences of Ghent University.

Intranasal instillation of reagents

DEP (SRM 2975) was purchased from the National Institute for Standards and Technology (NIST). HDM (Dermatophagoides pteronyssinus) was obtained from Greer laboratories. Saline, 1µg HDM extract dissolved in saline, 25µg DEP suspended in saline or a combination of DEP+HDM were delivered intranasally to isoflurane anesthetized mice using a continuous flow vaporizer on day 1, 8 and 15. Two days after the last challenge, mice were sacrificed with a lethal dose intraperitoneal pentobarbital. Tween 80 (0.05%) was used as a vehicle in all conditions.

Bronchoalveolar lavage fluid (BALF)

A tracheal cannula was inserted and BALF was recuperated by instillation of 3 x 300µl 1% Hank's balanced salt solution (HBSS) supplemented with 1% bovine serum albumin (BSA) and 6 x 500µl HBSS supplemented with ethylenediaminetetraacetic acid (EDTA). The lavage fractions were pooled and total cell counts were measured using a Bürker chamber. Differential cell counts were performed on cytospin after a May-Grünwald-Giemsa staining. The remaining cells were used for flow cytometry.

Lung and mediastinal lymph node single cell suspensions

The pulmonary circulation was rinsed with saline supplemented with EDTA to remove the intravascular pool of cells. Lungs and mediastinal lymph nodes (MLN) were minced and incubated for 45 minutes in digestion medium (roswell park memorial institute medium (RPMI)-1640 supplemented with 5% foetal calf serum (FCS), 2 mM L-glutamine, 0.05 mM 2-mercaptomethanol,

100 U/ml penicillin, 100 mg/ml streptomycin, 1 mg/ml collagenase type 2, and 0.02 mg/ml DNase I) at 37°C and 5% CO2. Red blood cells were lysed using ammonium chloride buffer. Total cell counts were performed with a Z2 Coulter Counter.

Mediastinal lymph node cell culture

MLN were harvested and digested as described above. Cells were cultured in culture medium either alone or supplemented with 3.75µg/well HDM in round bottom, 96-well plates and incubated in a humidified 37°C incubator with 5% CO2. After 5 days, supernatants was harvested for cytokine measurements.

Flow cytometry

BALF cells and single lung suspensions were stained with a combination of anti-mouse fluorochrome-conjugated monoclonal antibodies against: CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD69 (H1.2F3), Ly6C (AL-21), Ly6G (1A8), MHCII (2G9), Siglec-F (E50-2440) (all BD Biosciences); CD3 (145-2C11), CD90.2 (30.H12) (all Biolegend); CD5 (53-7.3), CD11c (N418), CD25 (PC61.5), CD127 (A7R34), CD45R (RA3-6B2), NK1.1 (PK136), TCRβ (H57-597) (all eBioscience). For cytoplasmatic cytokine staining, cells were stimulated for 4 hours with ionomycin and phorbol 12-myristate 13-acetate (PMA), supplemented with brefeldin A and monensin at 37°C for 4h. The intracellular fixation and permeabilization buffer set (eBioscience) was used for fixation and cell permeabilization. The following antibodies were used: PE-conjugated anti-IL-5 (TRFK5), anti-IL-13 (eBio13A) and isotype-matched controls (eBioscience). Data acquisition was performed on a FACSCalibur flow cytometer running CellQuest software or a LSR II cytometer running DiVa software. 250.000 events were collected. Cell subsets were analyzed using FlowJo software. Representative flow cytometric density plots and gating strategy of all analyzed cell populations in BALF and lung tissue can be found in the materials & methods section (Figure 15, p.54).

Histology

The left lung was fixated with 4% paraformaldehyde (PFA) and embedded in paraffin. 3µm transversal sections were stained with a Congo Red staining to identify eosinophils or with a Periodic Acid-Schiff (PAS) staining to analyze goblet cells. Quantitative measurements were performed using a Zeiss KS400 Image analyzer platform.

Protein measurements

IL-4, IL-5 and IL-13 levels in BALF or MLN supernatants were measured by commercially available ELISA kits (R&D systems). In lung homogenates, IL-25 and IL-33 were measured with ELISA (R&D systems) following the manufacturer's instructions. HDM-specific IgG1 was determined on serum collected by retro-orbital bleeding. For detection, plates were coated with HDM extract. Serum was added, followed by an HRP-conjugated polyclonal goat anti-mouse IgG1 antibody (Bethyl Laboratories). The plate was read at 490nm. HDM-IgG1 levels were reported in optical densities (OD). All samples were on the same plate and experimental data from different plates are not co-presented.

Airway hyperresponsiveness (AHR)

Airway hyperresponsiveness in response to increasing doses carbachol (0, 20, 40, 80, 160, 320, 640 μg/kg) was measured 48 hours after the last intranasal instillation using the forced oscillation technique (Flexivent System, SCIREQ, Montreal, QC, Canada). Neuromuscular blockade was induced by injecting 1mg/kg pancuronium bromide intravenously. A "snapshot perturbation" maneuver was imposed to measure the resistance (R) of the whole respiratory system (airways, lung and chest wall).

Statistical analysis

Statistical analysis was performed with SPSS, version 22.0. Non parametric tests (Kruskal-Wallis and Mann-Whitney-U) were used to compare different groups, according to the standard statistical criteria. Values were reported as mean ± SEM. P-values < 0.05 were considered as significant.

RESULTS

Exposure to diesel exhaust particles enhances house dust mite-induced airway inflammation

C57BL/6 mice were exposed to saline, DEP or HDM alone, or combined DEP+HDM **(Fig. 1A)**. In order to have a model wherein we could optimally examine the potential adjuvant capacities of DEP on HDM-induced airway inflammation, mice were exposed to doses of DEP and HDM that elicited almost no inflammatory response on their own (dose titrations for HDM are shown in **Fig. S1**, BALF inflammation to low versus high doses of DEP was previously shown [94]). Exposure to DEP alone slightly increased the amount of DC, neutrophils and $CD4^+$ T cells in the BALF, in comparison with saline-exposed mice **(Fig. 1D, E and G)**. Administration of HDM induced a modest increase of DC, $CD4^+$ T cells, $CD8^+$ T cells and eosinophils in the BALF when compared with the saline group **(Fig. 1D, G, H and I)**. In contrast, combined exposure to DEP+HDM greatly enhanced the allergic airway immune responses. Concomitant exposure to DEP+HDM elicited a marked increase in the epithelial-derived cytokines IL-25 and IL-33 **(Fig. 1B, C)**. Moreover, the numbers of DC, neutrophils, ILC2, $CD4^+$ T cells, $CD8^+$ T cells and eosinophils were significantly increased in the BALF of WT mice that received combined DEP+HDM compared with the three control groups **(Fig. 1D-I)**. Of note, all ILC2 expressed ST2 (data not shown), resembling natural ILC2 [208]. Co-exposure of DEP+HDM was also associated with a significant enhancement of inflammatory cells in the lung tissue **(Fig. S2)**. Furthermore, histological examination revealed increased peribronchovascular eosinophilic inflammation and goblet cell metaplasia upon simultaneous DEP+HDM exposure **(Fig. 2A, B)**.

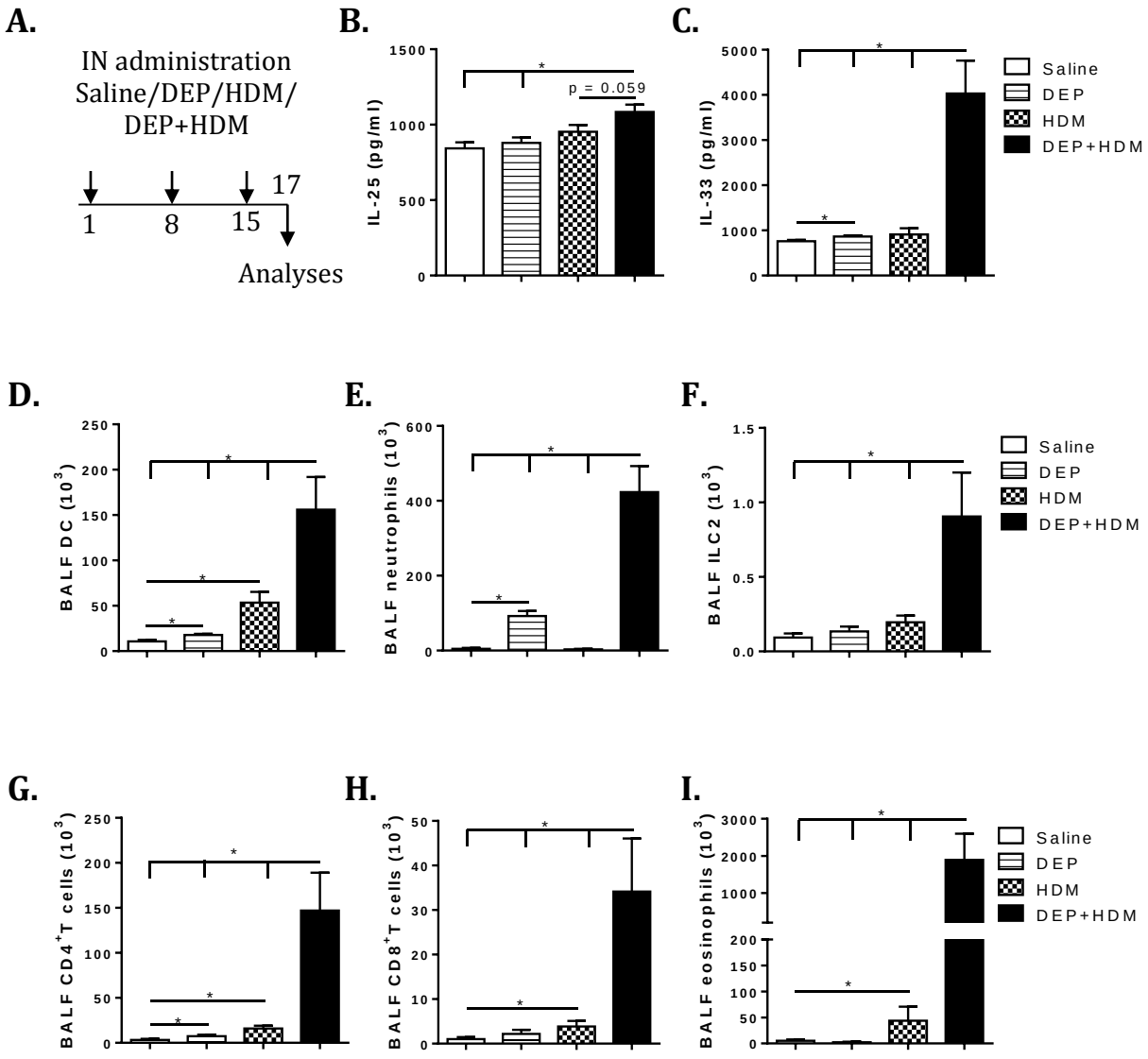


Figure 1: Exposure to diesel exhaust particles enhances house dust mite-induced airway inflammation in the BALF. WT mice were exposed to saline (white bar), 25µg DEP (striped bar), 1µg HDM (checked bar) or DEP+HDM (black bar) on day 1, 8 and 15. **A,** Schematic overview of our model of DEP-enhanced allergic airway inflammation. **B-C,** IL-25 **(B)** and IL-33 **(C)** protein levels in lung were determined by ELISA. **D-I,** DC ($CD11c^{high}$, low autofluorescent, $MHCII^{+}$) **(D)**, neutrophils **(E)**, ILC2 (Lin^{-} ($CD3^{-}$, $CD5^{-}$, $NK1.1^{-}$, $TCR\beta^{-}$, $CD11c^{-}$, $CD11b^{-}$ and $CD45R^{-}$) $CD25^{+}$ $CD90^{+}$) **(F)**, $CD4^{+}$ T cells ($CD3^{+}$ $CD4^{+}$ $CD8^{-}$) **(G)**, $CD8^{+}$ T cells ($CD3^{+}$ $CD8^{+}$ $CD4^{-}$) **(H)** and eosinophils **(I)** in BALF were determined by flow cytometry except neutrophils and eosinophils that were determined on cytospin. Results are expressed as mean ± SEM. n = 7-8 mice per group. * $p < 0.05$. Data are representative of three independent experiments. Representative flow cytometric plots and gating strategy can be found in the materials and methods (Fig.15, p54).

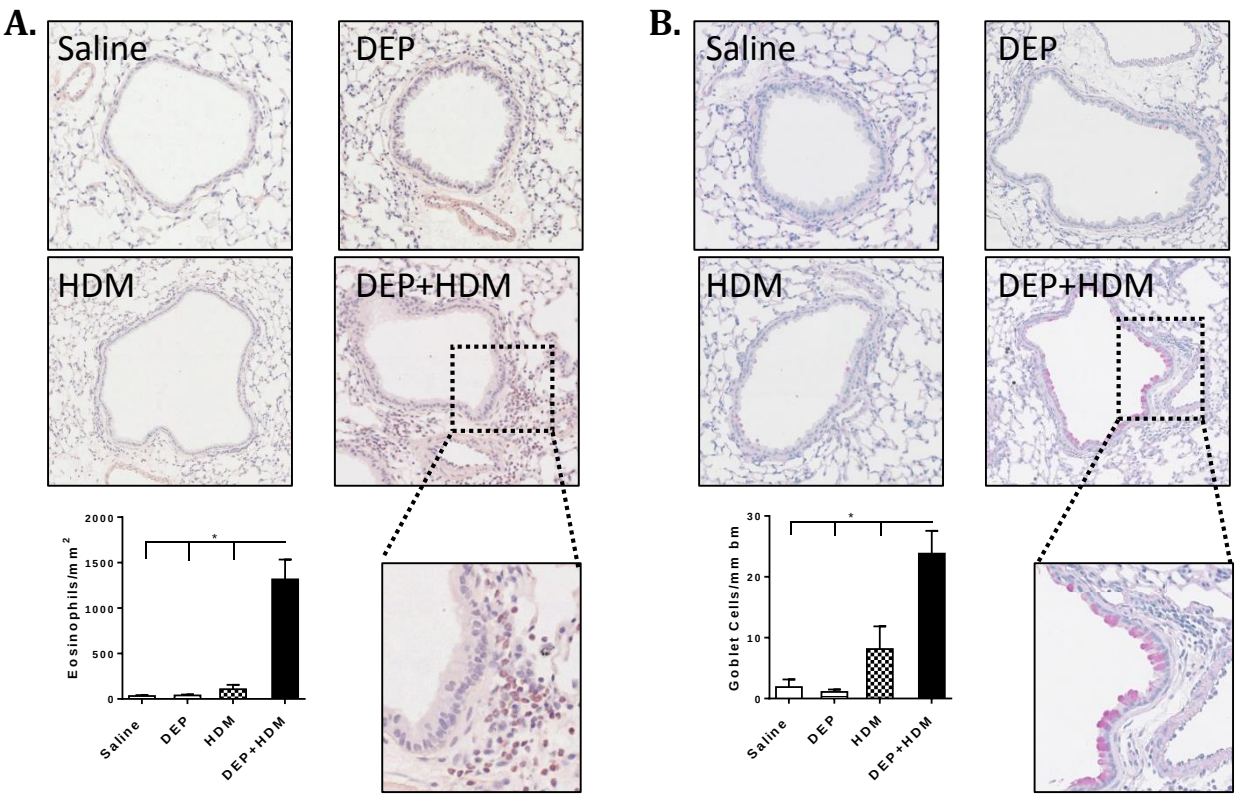


Figure 2: Exposure to diesel exhaust particles enhances house dust mite-induced airway inflammation in the lung. WT mice were exposed to saline (white bar), 25µg DEP (striped bar), 1µg HDM (checked bar) or DEP+HDM (black bar) on day 1, 8 and 15. **A-B,** Representative photomicrographs and quantification of peribronchovascular eosinophils **(A)** and PAS-stained lung specimen **(B)**. Results are expressed as mean ± SEM. n = 7-8 mice per group. * $p < 0.05$. Data are representative of three independent experiments.

Combined exposure to DEP+HDM increases type 2 cytokine production, HDM-specific IgG1 and induces airway hyperresponsiveness

Typical type 2 cytokines were evaluated in the BALF and supernatant of HDM-restimulated MLN that were obtained from WT mice exposed to saline, DEP or HDM alone, or combined DEP+HDM. In the BALF of mice exposed to DEP+HDM, elevated IL-5 and IL-13 levels were found compared to the three control groups, whereas HDM or DEP alone elicited no response **(Fig. 3A, B)**. In the supernatant of HDM-restimulated MLN, concomitant DEP+HDM exposed mice had marked higher levels of IL-4, IL-5 and IL-13, compared to the control groups **(Fig. 3C-E)**. In contrast, exposure to DEP alone was associated with a modest increase in IL-4 and IL-13 in comparison with the saline group **(Fig. 3C, E)**. Sole HDM administration slightly increased IL-5 and IL-13 levels in the MLN compared with the saline-exposed group **(Fig. 3D, E)**. Furthermore, combined DEP+HDM exposed mice had significantly elevated HDM-specific IgG1 titers in serum, when compared to saline-, DEP- and HDM-exposed control groups **(Fig. 3F)**. Additionally, mice that were concomitantly exposed to DEP+HDM showed AHR in comparison with the three control groups **(Fig. 3G)**.

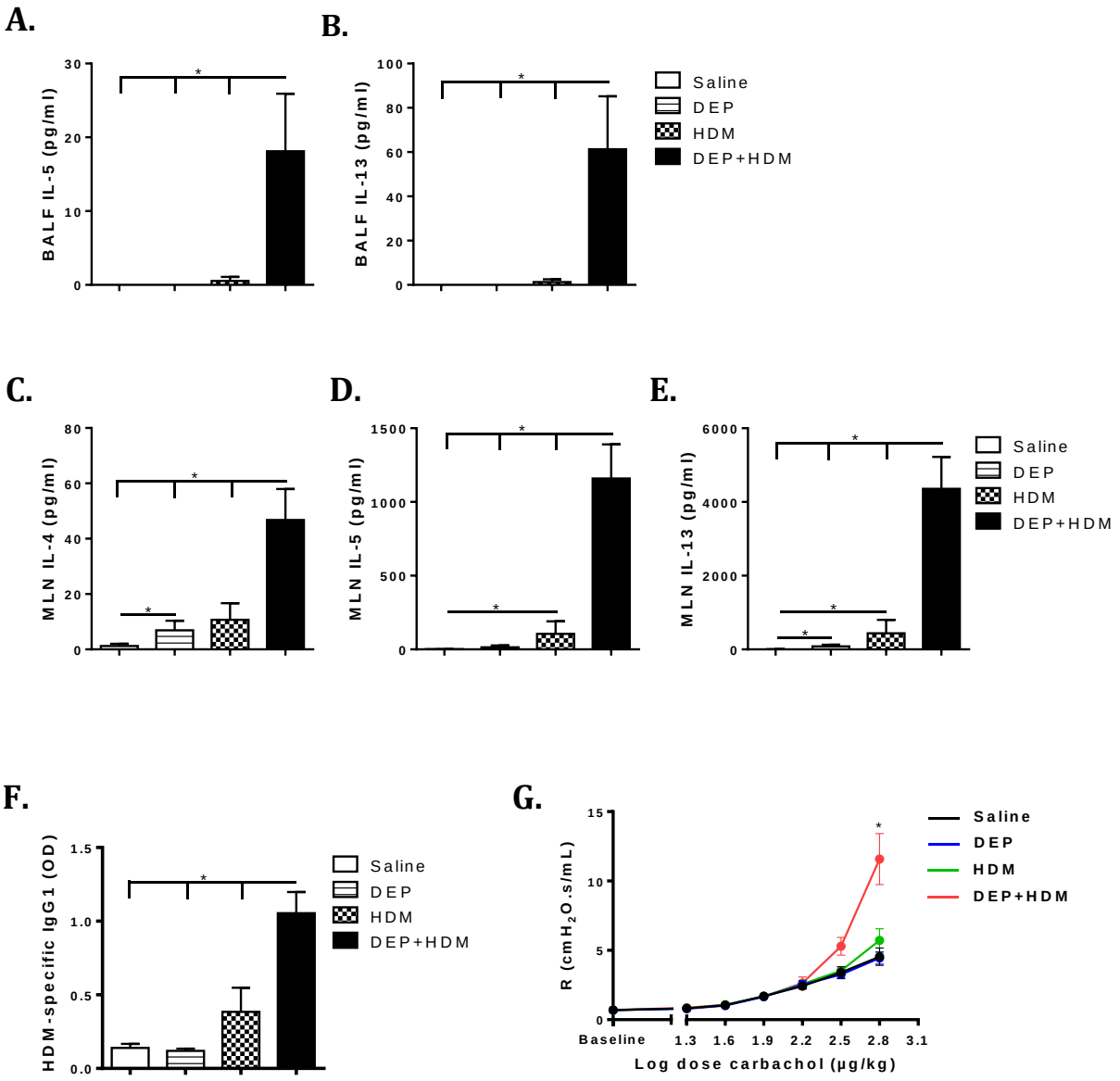


Figure 3: Combined exposure to DEP+HDM increases type 2 cytokine production, HDM-specific IgG1 and induces airway hyperresponsiveness. WT mice were exposed to saline (white bar), 25µg DEP (striped bar), 1µg HDM (checked bar) or DEP+HDM (black bar) on day 1, 8 and 15. **A-B,** IL-5 **(A)** and IL-13 **(B)** protein levels in BALF were determined by ELISA. **C-E,** IL-4 **(C)**, IL-5 **(D)**, IL-13 **(E)** protein levels in the supernatants of HDM-restimulated MLN were determined by ELISA. **F,** HDM-specific IgG1 titers in serum were determined by ELISA. **G,** Airway resistance (R) of saline (black line), DEP (blue line), HDM (green line) and DEP+HDM (red line) exposed mice was measured in response to increasing doses of carbachol. Results are expressed as mean ± SEM. n = 7-8 mice per group. * $p < 0.05$. Data are representative of two independent experiments.

Reduced Gata-3 expression impairs airway eosinophilia and mucus metaplasia upon combined DEP+HDM exposure

Gata-3 is an important transcription factor for the development of ILC2 and T_H2 cells [315]. Since the Gata-3 gene copy number is positively correlated with both ILC2 and T_H2 function [318-320], we evaluated the effect of reduced Gata3 expression in our model of DEP-enhanced allergic airway inflammation. $Gata3^{+/nlslacZ}$ mice, in which one allele is disrupted by insertion of a beta-

galactosidase reporter [316], and WT littermates were exposed to saline, DEP or HDM alone, or the combination of DEP+HDM. Concomitant DEP+HDM exposure in Gata3$^{+/nlslacZ}$ mice resulted in DC, neutrophil, CD4^{+} T cell and CD8^{+} T cell numbers that were comparable to those in WT littermates, whereas ILC2 and eosinophil numbers in the BALF were significantly reduced **(Fig. 4A-F)**. Moreover, histological analyses in DEP+HDM exposed Gata3$^{+/nlslacZ}$ mice revealed a diminished peribronchial eosinophilic inflammation and goblet cell metaplasia compared to their littermate controls **(Fig. 5A, B)**. Also upon sole HDM exposure, a reduced eosinophilia **(Fig. 5A)** and goblet cell metaplasia **(Fig. 5B)** was observed in the Gata3$^{+/nlslacZ}$ mice in comparison with littermates. Comparable HDM-specific IgG1 levels in the serum were found in WT and Gata3$^{+/nlslacZ}$ mice that were exposed to DEP+HDM **(Fig. 5C)**. Moreover, concomitant exposure to DEP+HDM led to a similar increase in airway responsiveness in Gata3$^{+/nlslacZ}$ mice and WT littermates, when compared with their HDM control groups **(Fig. 5D)**.

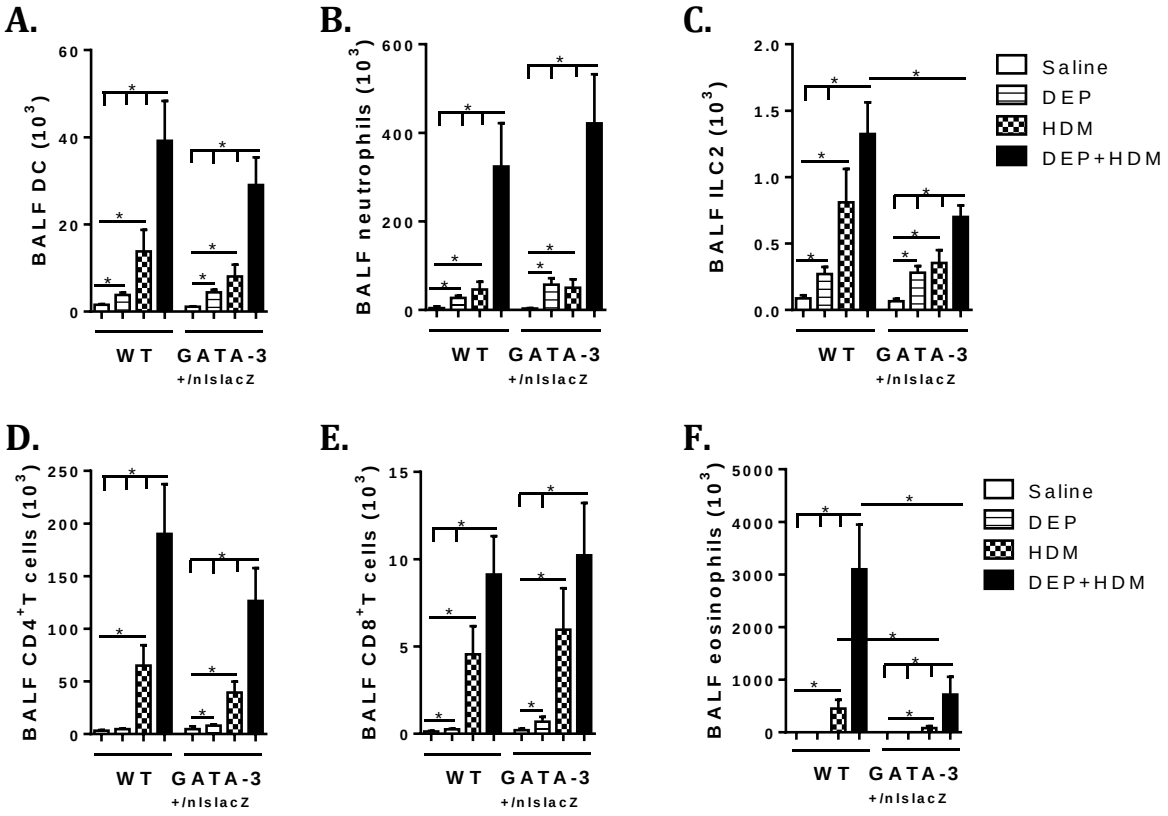


Figure 4: Reduced Gata-3 expression impairs BALF eosinophilia upon combined DEP+HDM exposure. WT and Gata3$^{+/nlslacZ}$ mice were exposed to saline (white bar), 25µg DEP (striped bar), 1µg HDM (checked bar) or DEP+HDM (black bar). **A-F,** DC (CD11c^{high}, low autofluorescent, MHCII^{+}) **(A)**, neutrophils **(B)**, ILC2 (Lin^{-} (CD3^{-}, CD5^{-}, NK1.1^{-}, TCRβ^{-}, CD11c^{-}, CD11b^{-} and CD45R^{-}) CD25^{+} CD90^{+}) **(C)**, CD4^{+} T cells (CD3^{+} CD4^{+} CD8^{-}) **(D)**, CD8^{+} T cells (CD3^{+} CD8^{+} CD4^{-}) **(E)** and eosinophils **(F)** in BALF were determined by flow cytometry except neutrophils and eosinophils that were determined on cytospin. Results are expressed as mean ± SEM. n= 7-9 mice per group. * $p < 0.05$.

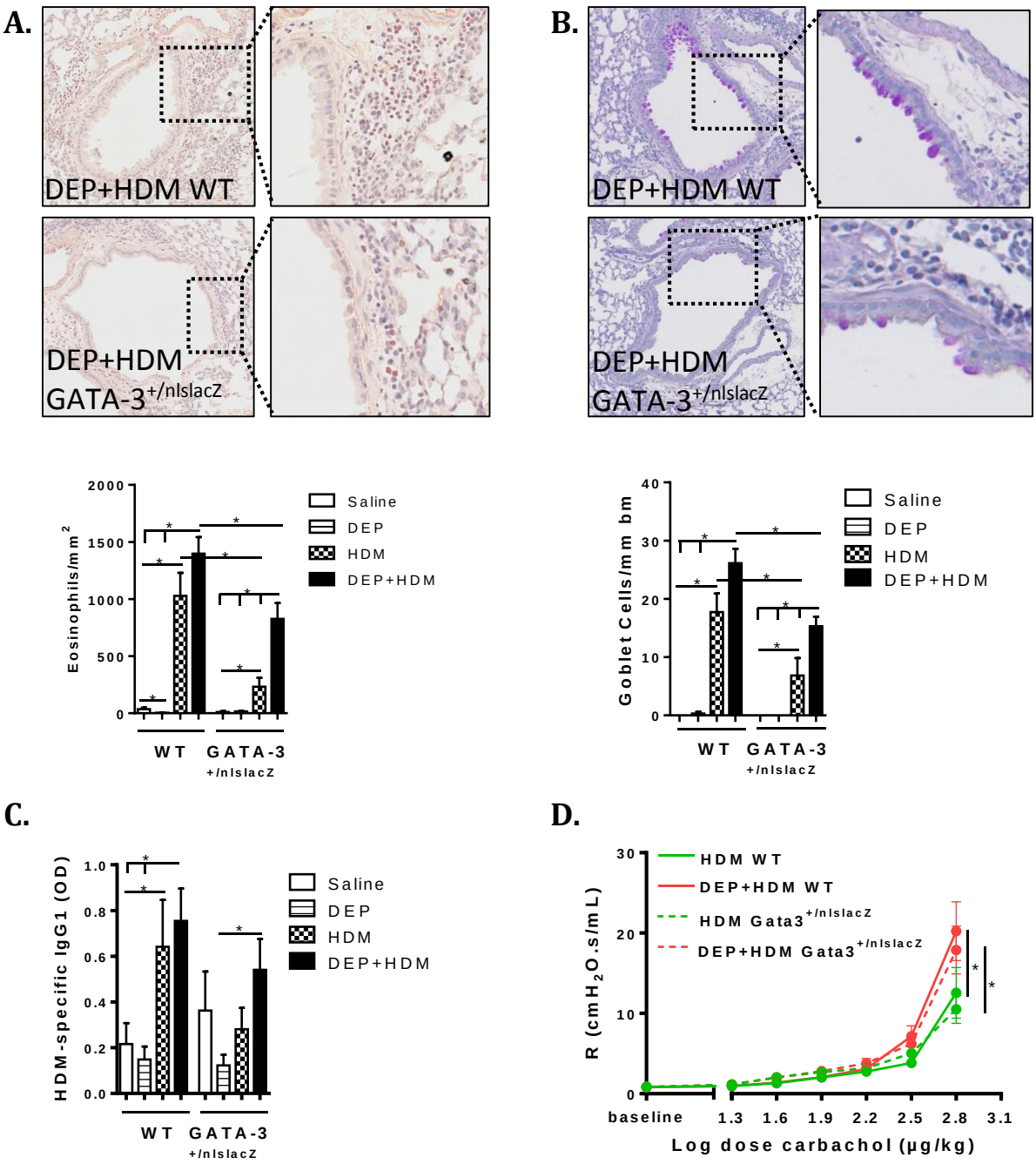


Figure 5: Reduced Gata-3 expression impairs airway eosinophilia and mucus metaplasia upon combined DEP+HDM exposure. WT and Gata3$^{+/nlslacZ}$ mice were exposed to saline (white bar), 25µg DEP (striped bar), 1µg HDM (checked bar) or DEP+HDM (black bar). **A-B,** Representative photomicrographs and quantification of congo red stained lungs **(A)** or PAS-stained mucus producing goblet cells **(B)** of DEP+HDM exposed WT and Gata3$^{+/nlslacZ}$ mice. **C,** HDM-specific IgG1 levels in serum were determined by ELISA. **D,** Airway resistance (R) in WT (full line) and Gata3$^{+/nlslacZ}$ (broken line) mice was measured in response to increasing doses of carbachol. Results are expressed as mean ± SEM. n= 7-9 mice per group. * $p < 0.05$.

Reduced Gata-3 expression decreases type 2 cytokine production by ILC2 and CD4$^+$ T cells upon combined DEP+HDM exposure

We further assessed the type 2 cytokine production in Gata3$^{+/nlslacZ}$ mice and WT littermates in response to combined DEP+HDM. Exposure to DEP+HDM led to reduced IL-13 levels in BALF of Gata3$^{+/nlslacZ}$ mice, compared to littermates **(Fig. 6A)**. In addition, intracellular type 2 cytokine production in both ILC2 and CD4$^+$ T cells was investigated in BALF and lung tissue. The increased numbers of IL-13 positive ILC2 and CD4$^+$ T cells that were observed in BALF of DEP+HDM WT mice were significantly decreased in DEP+HDM exposed Gata3$^{+/nlslacZ}$ mice **(Fig. 6B, C)**. Moreover, IL-5 and IL-13 expressing ILC2 were significantly diminished in lung single cell suspensions of Gata3$^{+/nlslacZ}$ mice independent of the exposure, compared to littermates **(Fig. 6D, E)**. The increased percentages of IL-5 and IL-13 positive CD4$^+$ T cells that were observed in the co-exposed DEP+HDM Gata3$^{+/nlslacZ}$ mice did not significantly differ from their WT controls. In contrast, Gata3$^{+/nlslacZ}$ mice exposed to sole HDM had significant lower IL-5 and IL-13 positive CD4$^+$ T cells in comparison with the littermates **(Fig. 6F, G)**.

ILC2 marginally contribute to DEP-enhanced allergic airway inflammation

Since Gata-3 haploinsufficiency affects both the innate and adaptive component, we next examined the specific contribution of ILC2 in the enhancing effects of DEP on allergic airway inflammation. For that, we exposed RORα$^{fl/fl}$IL7R^{Cre} (ILC2-deficient) mice [249] and their corresponding RORα$^{fl/fl}$IL7R$^{+/+}$ controls to HDM and DEP+HDM. As expected, ILC2 numbers in the BALF were abolished in RORα$^{fl/fl}$IL7R^{Cre} mice, independent of the exposure **(Fig. 7A)**. Concomitant DEP+HDM exposed RORα$^{fl/fl}$IL7R^{Cre} mice had significant reduced DC in the BALF **(Fig. 7B)**, whereas the BALF neutrophils, CD4$^+$ T cells, CD8$^+$ T cells and eosinophils tended to decrease compared to RORα$^{fl/fl}$IL7R$^{+/+}$ controls **(Fig. 7C-F)**. Histological analyses further revealed a similar peribronchial eosinophilia and goblet cell metaplasia between DEP+HDM exposed RORα$^{fl/fl}$IL7R^{Cre} mice and controls **(Fig. 8A, B)**. Moreover, the increased BALF IL-5 (data not shown) and IL-13 levels found upon DEP+HDM exposure in the BALF and supernatants of restimulated MLN did not differ between RORα$^{fl/fl}$IL7R^{Cre} mice and RORα$^{fl/fl}$IL7R$^{+/+}$ controls **(Fig. 8C, D)**. Levels of HDM-specific IgG1 were also comparable between DEP+HDM exposed RORα$^{fl/fl}$IL7R^{Cre} mice and their controls **(Fig. 8E)**.

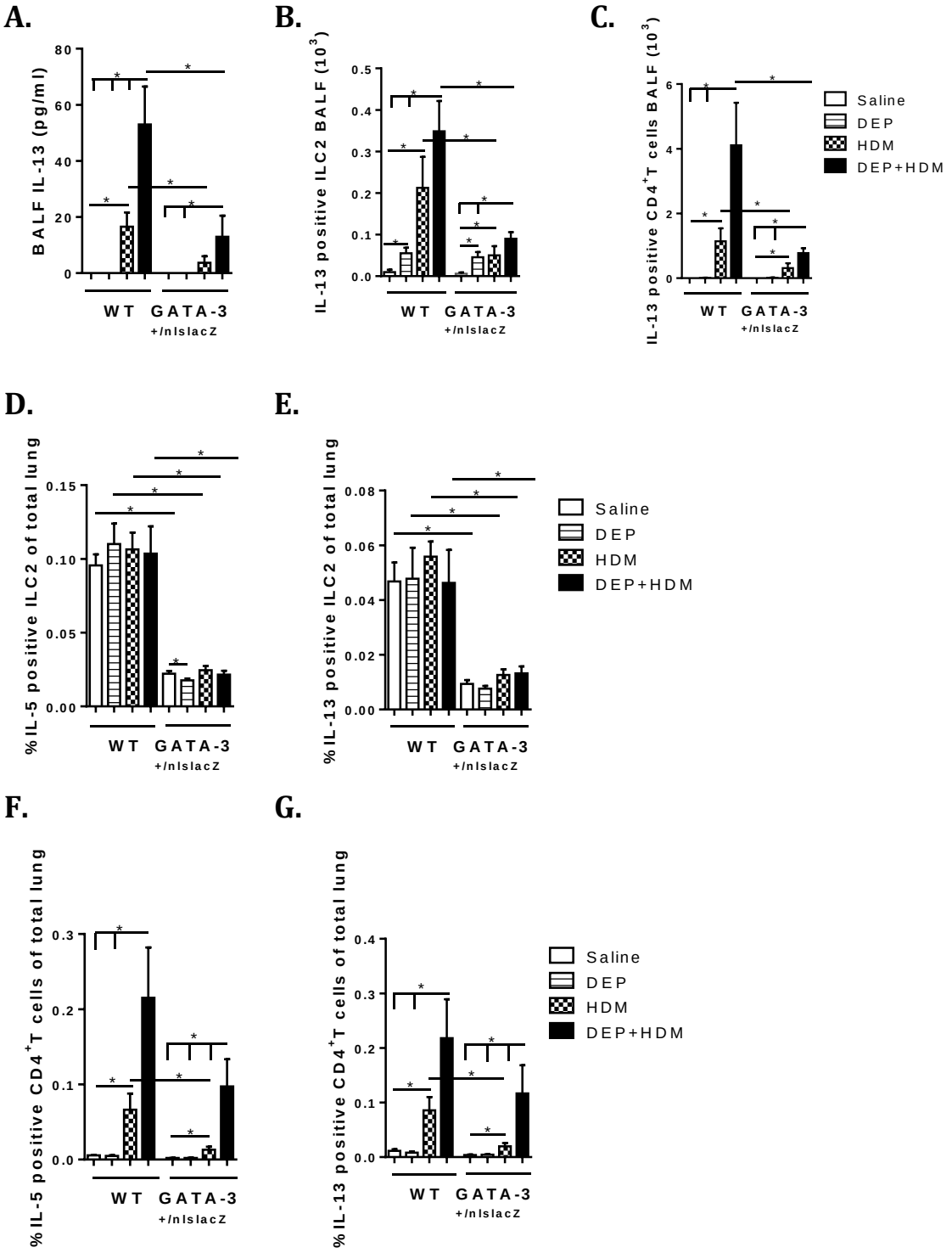


Figure 6: Reduced Gata-3 expression decreases cytokine production by ILC2 and CD4⁺ T cells upon combined DEP+HDM exposure. WT and Gata3$^{+/nlslacZ}$ mice were exposed to saline (white bar), 25µg DEP (striped bar), 1µg HDM (checked bar) or DEP+HDM (black bar). **A,** IL-13 protein levels in BALF were determined by ELISA. **B-G,** BALF or lung cells were stimulated for 4 hours with PMA/iomycin, intracellular labeled for cytokine production and analyzed using flow cytometry. Percentage IL-13 expressing ILC2 (Lin⁻ (CD3⁻, CD5⁻, NK1.1⁻, TCRβ⁻, CD11c⁻, CD11b⁻ and CD45R⁻) CD25⁺ CD90⁺) **(B)** or CD4⁺ T cells (CD3⁺ CD4⁺) **(C)** in BALF. Proportion of IL-5 **(D)** and IL-13 **(E)** producing ILC2 (Lin⁻ (CD3⁻, CD5⁻, NK1.1⁻, TCRβ⁻, CD11c⁻, CD11b⁻ and CD45R⁻) CD25⁺ CD90⁺) in the total lung tissue. Percentages IL-5 **(F)** and IL-13 **(G)** positive T cells (CD3⁺ CD4⁺) of total lung. Results are expressed as mean ± SEM. n = 7-8 mice per group. * $p < 0.05$. Representative flow cytometric histograms and density plots can be found in materials and methods (Fig.15, p.54)**.**

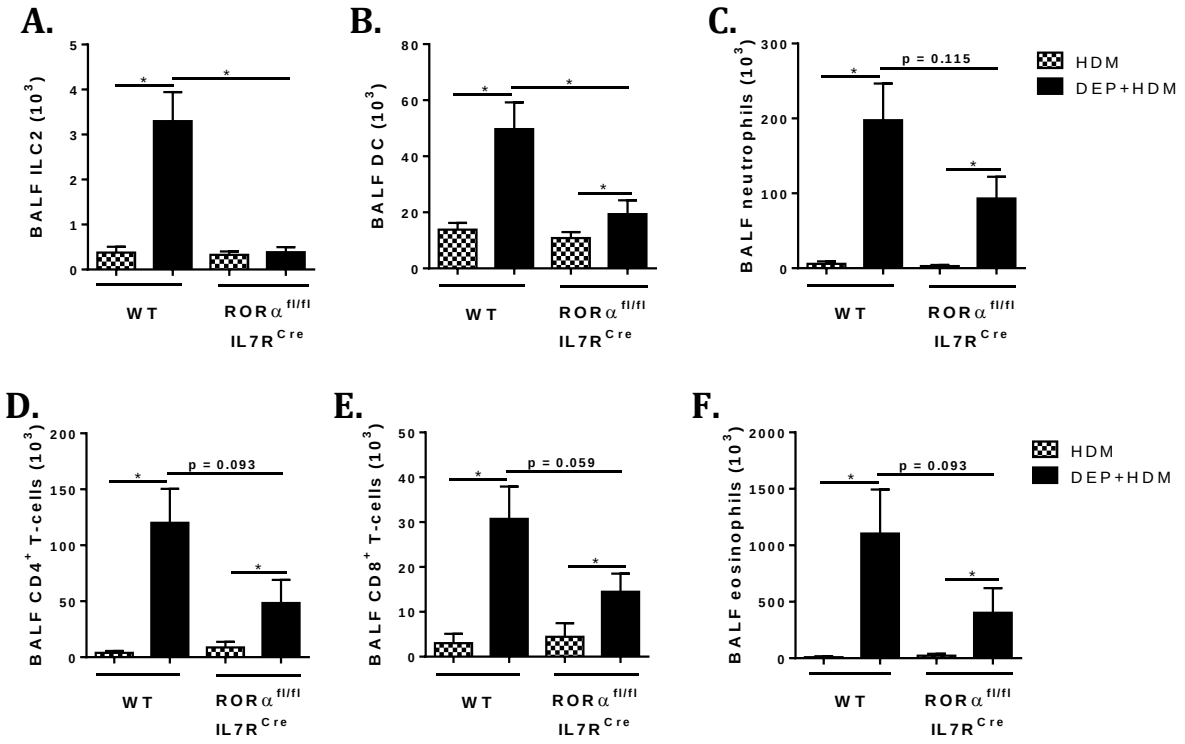


Figure 7: ILC2 marginally contribute to DEP-enhanced allergic airway inflammation. RORα$^{fl/fl}$IL7R^{Cre} mice and RORα$^{fl/fl}$IL7R$^{+/+}$ WT controls were exposed to 1µg HDM (checked bar) or DEP+HDM (black bar). **A-F,** ILC2 (Lin$^-$ (CD3$^-$, CD5$^-$, NK1.1$^-$, TCRβ$^-$, CD11c$^-$, CD11b$^-$ and CD45R$^-$) CD25$^+$ CD90$^+$) **(A)**, DC (CD11c^{high,} low autofluorescent, MHCII$^+$) **(B)**, neutrophils **(C)**, CD4$^+$ T cells (CD3$^+$ CD4$^+$ CD8$^-$) **(D)**, CD8$^+$ T cells (CD3$^+$ CD8$^+$ CD4$^-$) **(E)** and eosinophils **(F)** in BALF were determined by flow cytometry except neutrophils and eosinophils that were determined on cytospin. Results are expressed as mean ± SEM. n = 8 mice per group. * $p < 0.05$.

The adaptive immune system has a crucial role in DEP-enhanced allergic airway inflammation

To investigate how the adaptive immune system contributes to the DEP-enhanced allergic airway inflammation, we exposed WT and Rag2$^{-/-}$ mice – which lack an adaptive immune system [317] – to saline, DEP or HDM alone, or combined DEP+HDM. As expected, Rag2$^{-/-}$ mice had no mature CD4$^+$ T cells in the lung, whereas the proportion of lung ILC2 was increased, independent of the exposure **(Fig. 9A, B)**. Rag2$^{-/-}$ mice and WT controls that were co-exposed to DEP+HDM had similar ratios of DC and neutrophils in the BALF **(Fig. 9C, D)**. However, combined DEP+HDM exposure resulted in a complete abolishment of BALF eosinophils compared to the WT controls **(Fig. 9E)**. Histological analyses further revealed a severely reduced eosinophilia and abolished goblet cell metaplasia in the Rag2$^{-/-}$ mice that received combined DEP+HDM in comparison with WT controls **(Fig. 10A, B)**. Additionally, combined DEP+HDM exposure failed to increase BALF IL-5 (data not shown) and IL-13 levels in BALF and supernatants of restimulated MLN in Rag2$^{-/-}$ mice compared with their WT controls **(Fig. 10 C, D)**. The modest inflammation that was seen in the WT mice upon sole HDM exposure was also completely abolished in the Rag2$^{-/-}$ mice.

Independent of the exposure, there was no HDM-specific IgG1 production in Rag2$^{-/-}$ mice, compared to WT controls (data not shown). Furthermore, RAG2$^{-/-}$ mice had no increase in airway responsiveness in response to DEP + HDM **(Fig. 10E)**.

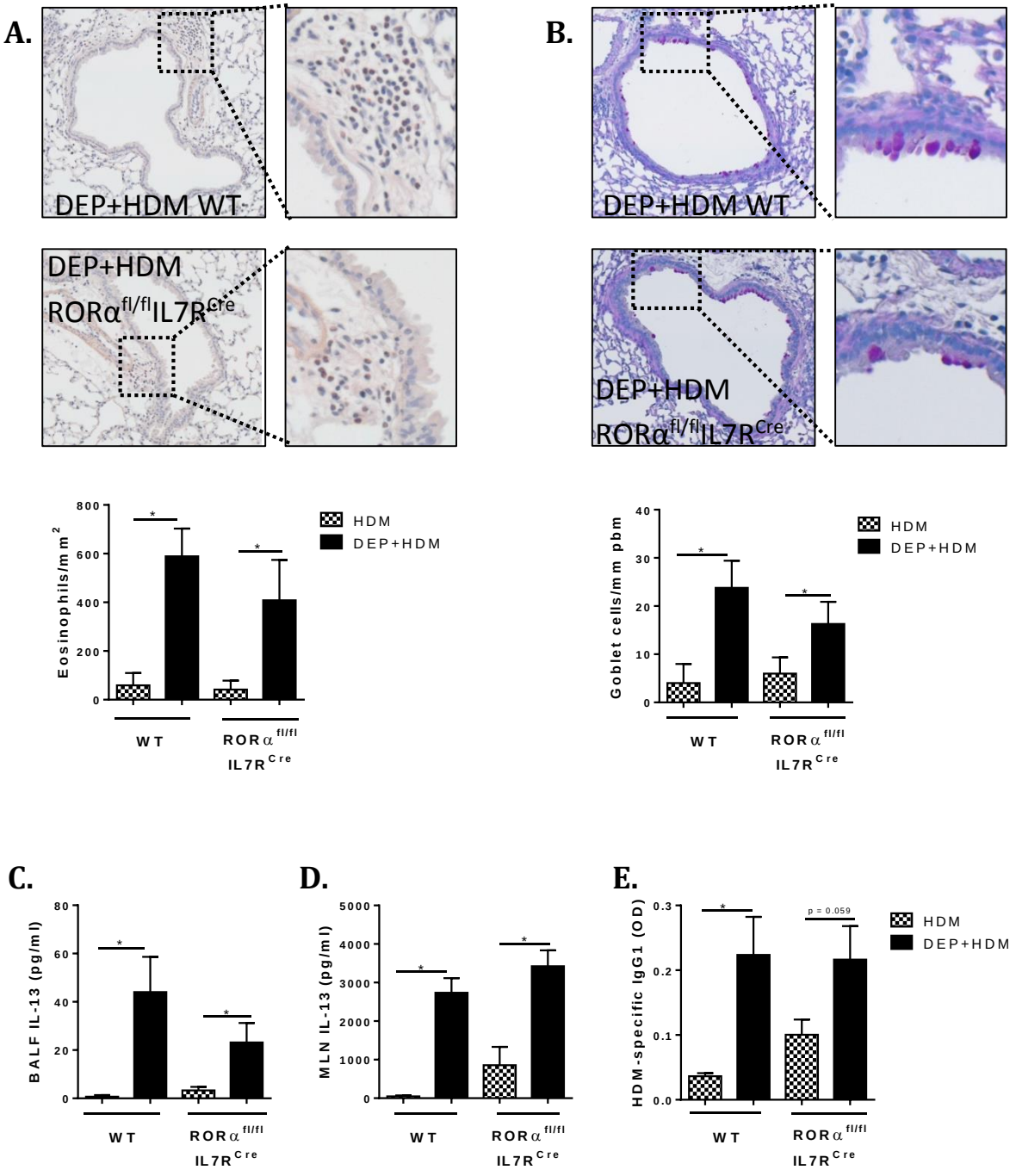


Figure 8: ILC2 marginally contribute to DEP-enhanced allergic airway inflammation. RORα$^{fl/fl IL7RCre}$ mice and RORα$^{fl/fl IL7R+/+}$ WT controls were exposed to 1µg HDM (checked bar) or DEP+HDM (black bar). **A-B,** Representative photomicrographs and quantification of congo red stained lungs **(A)** or PAS-stained mucus producing goblet cells **(B)**. **C-E,** BALF IL-13 protein levels **(C)**, IL-13 levels in the supernatants of HDM-restimulated MLN **(D)** and HDM-specific IgG1 levels in serum **(E)** were determined by ELISA. Results are expressed as mean ± SEM. n = 8 mice per group. * $p < 0.05$.

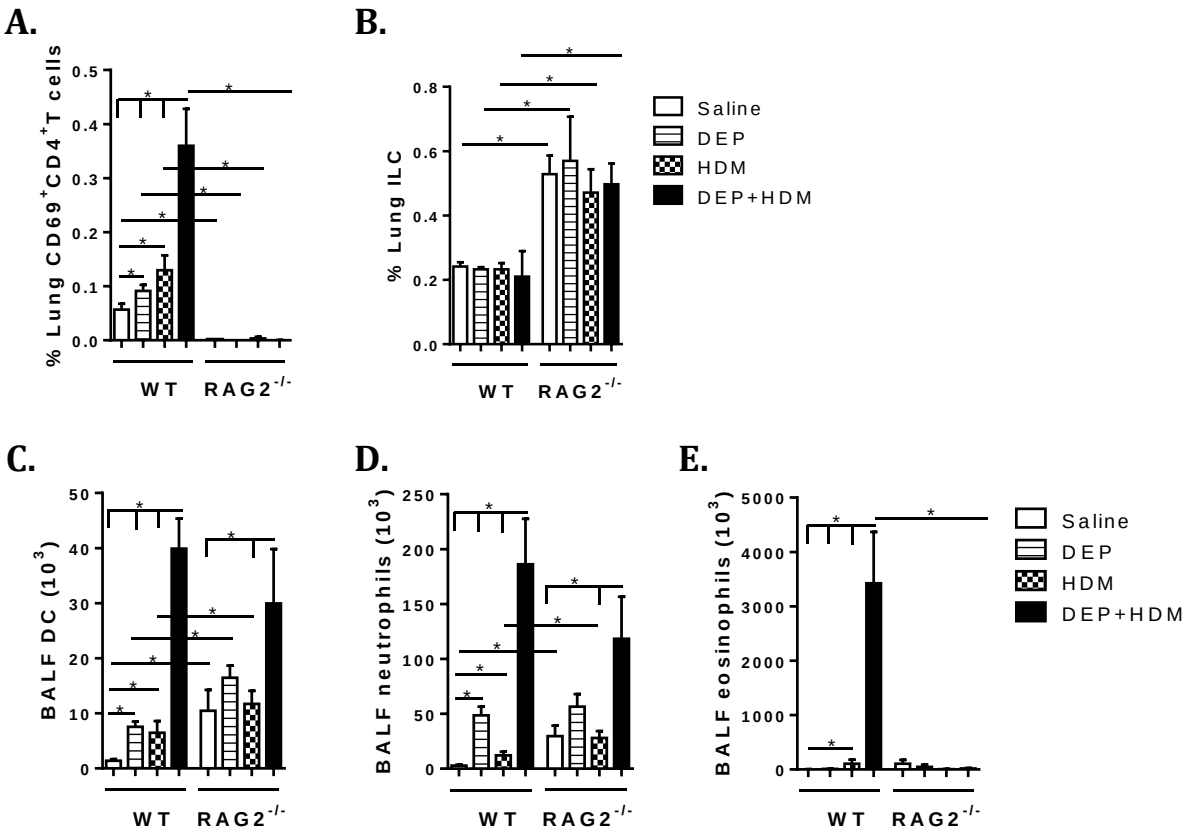


Figure 9: The adaptive immune system has a crucial role in DEP-enhanced allergic airway inflammation. WT and Rag2$^{-/-}$ mice were exposed to saline (white bar), 25µg DEP (striped bar), 1µg HDM (checked bar) or DEP+HDM (black bar). **A-E,** Percentage CD4^{+} T cells (CD3^{+} CD4^{+} CD8^{-} CD69^{+}) of total lung **(A)**, percentage lung ILC2 (Lin^{-} (CD3^{-}, CD5^{-}, NK1.1^{-}, TCRβ^{-}, CD11c^{-}, CD11b^{-} and CD45R^{-}) CD25^{+} CD127^{+}) **(B)**, number of BALF DC (CD11c^{high}, low autofluorescent, MHCII^{+}) **(C)**, number of BALF neutrophils **(D)** and number of BALF eosinophils **(E)** were determined by flow cytometry except neutrophils and eosinophils that were determined on cytospin. Results are expressed as mean ± SEM. n = 7-8 mice per group. * $p < 0.05$.

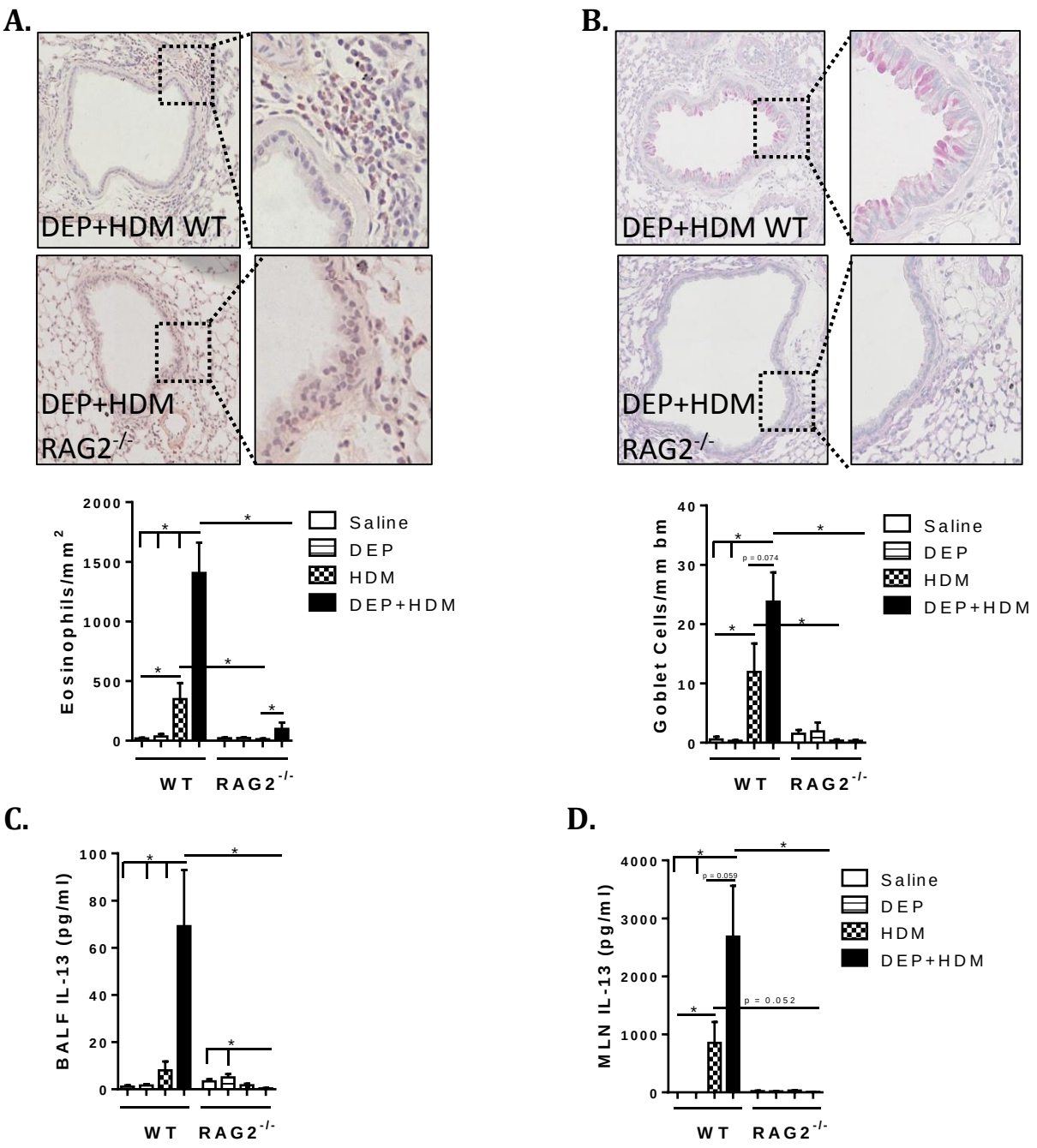


Figure 10: The adaptive immune system has a crucial role in DEP-enhanced allergic airway inflammation. WT and Rag2$^{-/-}$ mice were exposed to saline (white bar), 25µg DEP (striped bar), 1µg HDM (checked bar) or DEP+HDM (black bar. **A-B,** Representative photomicrographs and quantification of congo red stained lungs **(A)** or PAS-stained mucus producing goblet cells **(B)** of DEP+HDM exposed WT and Rag2$^{-/-}$ mice. **C-D,** BALF IL-13 protein levels **(C)** and IL-13 levels in the supernatants of HDM-restimulated MLN **(D)** were determined by ELISA. **E,** airway resistance (R) in WT (full line) and Rag2$^{-/-}$ (broken line) mice was measured in response to increasing doses of carbachol. Results are expressed as mean ± SEM. n = 7-8 mice per group. * $p < 0.05$.

DISCUSSION

In this paper, we demonstrated that dysregulation in ILC2 and T_H2 cell numbers and function by targeting Gata-3 was associated with an attenuated airway inflammation upon concomitant DEP+HDM exposure. Moreover, we showed a critical role of the adaptive immune system to DEP-enhanced allergic responses and AHR, whereas ILC2 only marginally contribute to DEP-enhanced allergic airway inflammation.

The immunological mechanisms by which DEP can promote allergen-induced airway inflammation are largely unknown. To unravel these cellular and molecular mechanisms, we set up a mouse model with concomitant exposure to a clinical relevant allergen (i.e. HDM) and DEP. To have an optimal model wherein the potential adjuvant effects of DEP on HDM-induced allergic airway inflammation can be evaluated, we downtitrated the dose of DEP and HDM until they elicited minimal inflammatory responses on their own. We demonstrated that concomitant exposure to DEP+HDM markedly enhanced multiple features of allergic inflammation, characterized by an eosinophilic response, goblet cell metaplasia, ILC2 and T_H2 cell accumulation, type 2 cytokine production, elevated HDM-specific IgG1 levels and AHR. In accordance with our findings, several experimental and epidemiological studies already provided evidence regarding the synergistic ability of DEP on allergic airway inflammation [23, 96, 99, 314]. Although these studies already demonstrated the enhanced effects on airway remodeling, AHR, eosinophilic inflammation and immunoglobulin production, insights concerning the number and function of the recently identified ILC2s in response to concomitant exposure to DEP+HDM were lacking.

Epithelial cells are the first barrier to encounter several inhaled allergens and particles. In response to these particulates, the epithelium can release cytokines and chemokines to direct the recruitment and activation of several innate and adaptive immune cells [102, 306]. Upon HDM for instance, it was shown that IL-25 and IL-33 levels were upregulated, contributing to the observed airway and lung inflammation [159, 321]. Although we observed no increased IL-25 and IL-33 response upon sole DEP or HDM, combined DEP+HDM exposure elicited an increase of IL-25 and IL-33 in particular, suggesting that DEP works synergistically with HDM to induce release of epithelial cytokines in the environment.

These epithelial-derived cytokines share the capacity to stimulate T_H2 development by polarizing DC on the one hand [102] and to activate ILC2 on the other hand [307, 308]. Whereas the role of T_H2 cells in the pathogenesis of asthma is well established [5], it was recently shown that ILC2 can also substantially contribute to allergic airway inflammation [311]. Moreover, ILC2 can mediate AHR, independent of the adaptive immune system [242, 312]. Importantly, we found that

exposure to DEP+HDM increased the number of cytokine expressing ILC2 and T_H2 cells in the alveolar space, suggesting that type 2 cytokine production of both cell types contributes to the adjuvant effects of DEP on allergic airway inflammation. Although the numbers of type 2 expressing ILC2 in the lung did not increase upon combined DEP+HDM exposure, ILC2 could still be critical in initiating and maintaining the DEP-enhanced type 2 immune responses. At least in response to the pollutant ozone, it was suggested that the increased activation of ILC2 was associated with an enhanced eosinophilic inflammation towards Aspergillus fumigates [255].

Since Gata-3 is an important transcription factor during the development and function of ILC2 as well as T_H2 cells [315], we investigated the effects of Gata-3 modulation in our model of DEP-enhanced allergic airway inflammation. At baseline, $Gata3^{+/nlslacZ}$ mice[4] had lower functional ILC2 in the lung, while effects on T_H2 cells were limited. In response to DEP+HDM, both IL-13 expressing ILC2 and T_H2 cells were attenuated in BALF of $Gata3^{+/nlslacZ}$ mice, suggesting that the observed reduction in type 2 airway inflammation could be the result of diminished functional ILC2 and T_H2 cells in the bronchoalveolar space. In addition, considering that co-exposure of DEP+HDM only tended to decrease the type 2 cytokine expressing T_H2 cells in the lung whereas the ILC2 were abolished, the imbalance of functional ILC2 and T_H2 cells in the lung could contribute to the reduced eosinophilic inflammation. Importantly, the reduction of type 2 expressing ILC2 and T_H2 cells in $Gata3^{+/nlslacZ}$ mice had no effect on the development of AHR in response to DEP+HDM[5]. Interestingly, the modest inflammation that was observed in response to only HDM was also greatly reduced in the $Gata3^{+/nlslacZ}$ mice. In line, exposure of Gata-3 mutant mice to ovalbumin inhibited the allergic airway inflammation [322]. Moreover, therapeutic targeting of Gata-3 in a clinical trial involving allergic asthmatics also attenuated both late and early allergen-induced asthmatic responses [323].

To further assess the relative contribution of the innate (i.e. ILC2) and adaptive (i.e. T_H2 cells) arm in our model of DEP-enhanced allergic airway inflammation, we used $ROR\alpha^{fl/fl}IL7R^{Cre}$ (ILC2-deficient) mice and $Rag2^{-/-}$ mice respectively. Intriguingly, we found that the DEP-enhanced allergic airway inflammation only tended to decrease in $ROR\alpha^{fl/fl}IL7R^{Cre}$ mice, whereas typical type 2 immune responses such as eosinophilia, mucus metaplasia and type 2 cytokine production were completely abolished in $Rag2^{-/-}$ mice that received combined DEP+HDM. In addition, $Rag2^{-/-}$ mice exposed to DEP+HDM failed to develop AHR. This suggests that the presence of an adaptive immune system or at least an adequate interaction of adaptive immune cells with ILC2 is required in mediating the adjuvant capacity of DEP on HDM-enhanced allergic airway inflammation and AHR. Of note, depletion of $CD4^+$ or $CD8^+$ T cells in a murine model with intraperitoneal exposure

to DEP+ovalbumin was associated with abrogated cytokine production and ovalbumin-specific immunoglobulin responses in the peritoneal exudate fluid [324]. Interestingly, whereas it was previously shown that ILC2 were critical in T-cell independent allergic lung inflammation [241], the modest inflammation in response to HDM in our model was completely abolished in the Rag2$^{-/-}$ mice. The amount of administered HDM could therefore be important, suggesting that doses of HDM that elicit limited biological inflammation on their own are unable to activate ILC2 and to drive the allergic airway inflammation in absence of an adaptive immune system. Furthermore, it could be that ILC2 are of less importance in subchronic responses than during the acute phases of an inflammatory response. This was supported by research performed in a papain model, where the secondary responses were more likely T_H2 dependent [170]. Moreover, it was suggested that the communication between ILC2 and $T_H2$cells by a MHCII-mediated dialog or specific cytokine secretion could be crucial to substantiate their effects on allergic airway inflammation [248, 249, 252]. It seemed however that upon concomitant DEP+HDM exposure activation of T_H2 cells appeared relatively independent of ILC2, opposed to previous reports where ILC2 were crucial for the initiation of T_H2 responses towards (relatively high) doses of HDM and papain [170, 245].

Taken together, although a significant role for ILC2s has been demonstrated in several models of allergen-induced inflammation, our findings suggest that co-exposure to multiple environmental factors, such as particulate pollutants and allergens (i.e. HDM), modulate the contribution of ILC2 and T_H2 cells to allergic airway inflammation and AHR.

SUPPLEMENTAL FIGURES

Figure S1: Titration of the HDM dose for the combined DEP+HDM model. C57BL/6 mice were exposed to decreasing doses of sole HDM (i.e. 12.5µg, 2.5µg, 1µg, 250ng, 25ng HDM) or combined 25µg DEP + decreasing doses of HDM on day 1, 8 and 15. On day 17, BALF eosinophils were determined on cytospin. Data presented on the different graph represent separate experiments. The red box represents the selected dose for the experiments in the manuscript. Results are expressed as mean ± SEM. n = 8 mice per group. * $p < 0.05$.

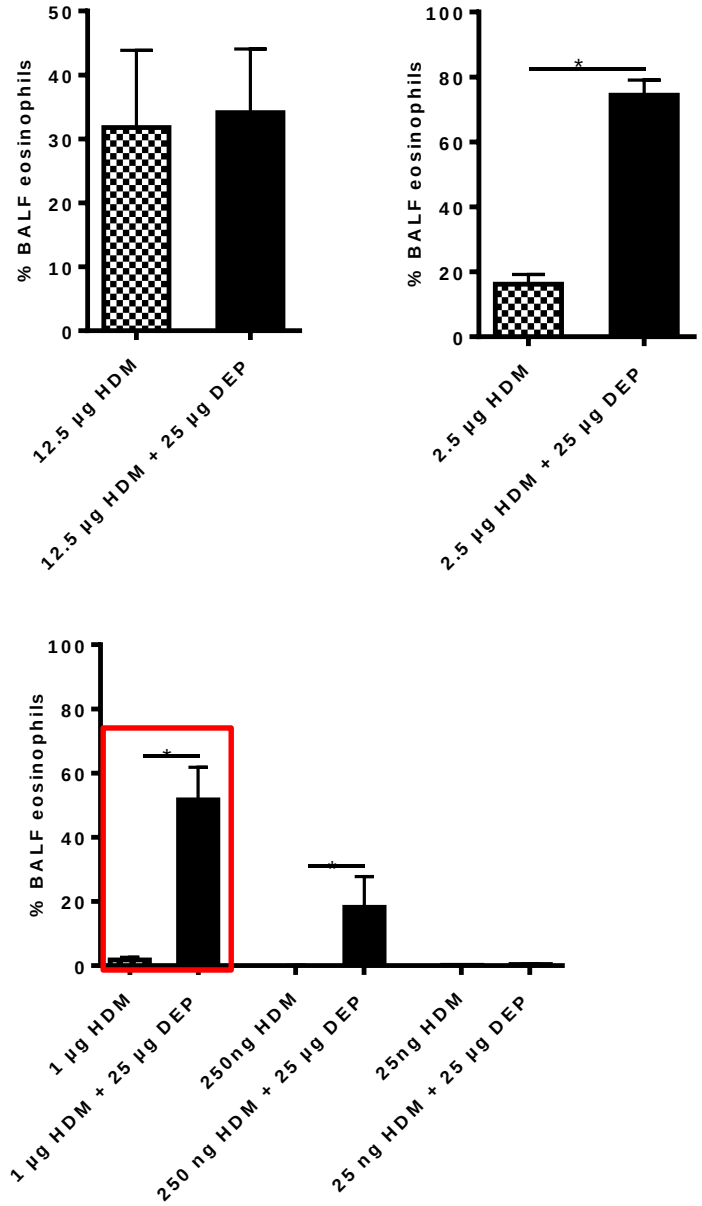
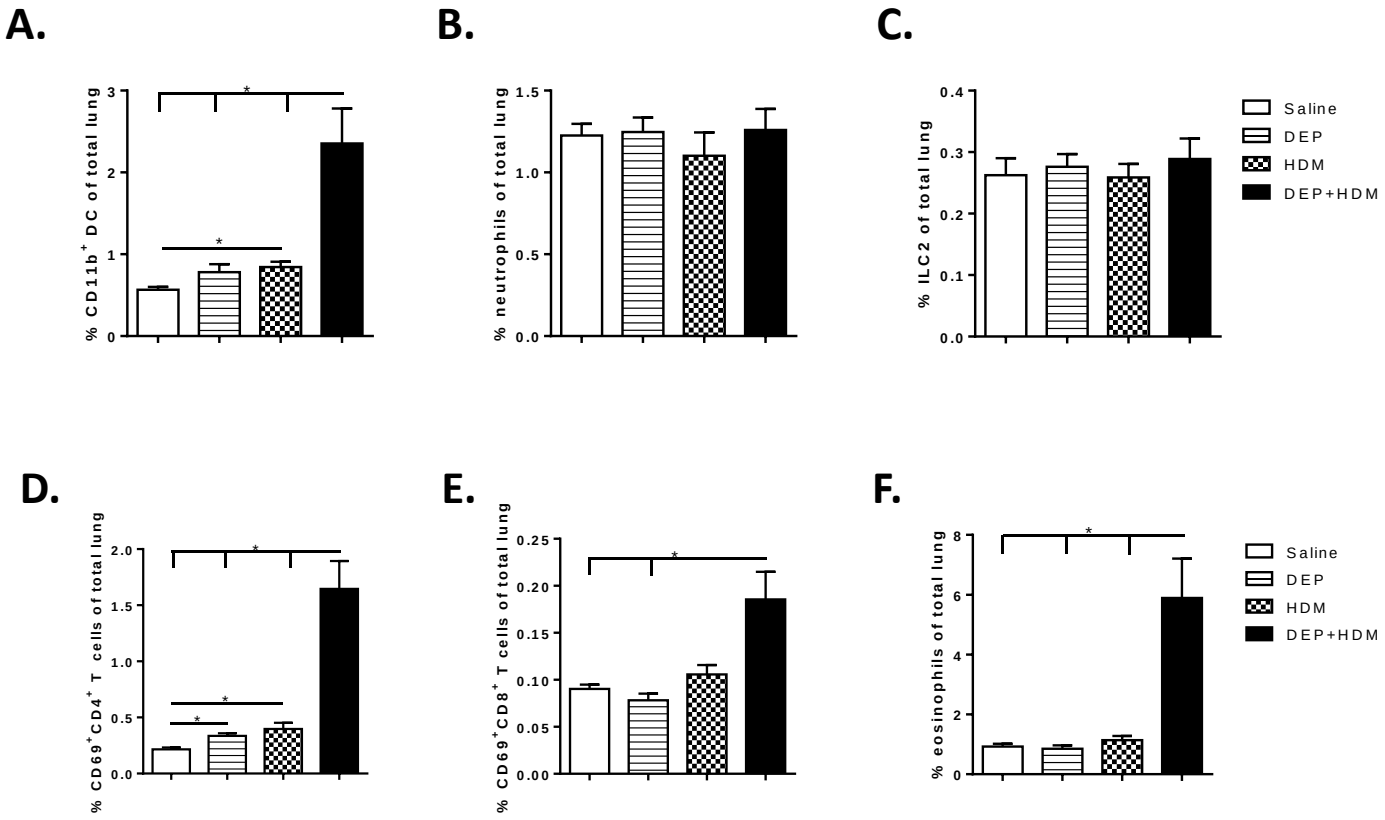


Figure S2: Adjuvant effect of DEP on HDM induced airway inflammation in the lung. WT mice were exposed to saline (white bars), 25µg DEP (striped bars), 1µg HDM (checked bars) or DEP+HDM (black bars) on day 1, 8 and 15. **A-F,** DC ($CD11c^+$ $MHCII^+$ $CD11b^+$) **(A)**, neutrophils ($CD11c^-$ $CD11b^+$ $Ly6G^+$ $Ly6C^+$) **(B)**, eosinophils ($CD11c^-$ $CD11b^+$ $Siglec\text{-}F^+$) **(C)**, ILC2 (Lin$^-$ ($CD3^-$, $CD5^-$, $NK1.1^-$, $TCR\beta^-$, $CD11c^-$, $CD11b^-$ and $CD45R^-$) $CD25^+$ $CD90^+$) **(D)**, $CD4^+$ T cells ($CD3^+$ $CD4^+$ $CD8^-$ $CD69^+$) **(E)** and $CD8^+$ T cells ($CD3^+$ $CD8^+$ $CD4^-$ $CD69^+$) **(F)** in lung tissue were determined by flow cytometry. Results are expressed as the mean percentages of total lung ± SEM. n = 7-8 mice per group. * $p < 0.05$.



CHAPTER 10: CHARACTERIZATION AND QUANTIFICATION OF INNATE LYMPHOID CELLS SUBSETS IN HUMAN LUNG

Innate lymphoid cells (ILCs) are important regulators of tissue homeostasis, injury and inflammation. Whereas human ILC have been extensively studies in the intestines and skin, limited data are available concerning ILCs in the human respiratory system. The aim of this study was to characterize the different ILC subsets in human lung tissue and to investigate their relative abundance in COPD patients.

De Grove KC*, Provoost S*, Verhamme FM, Bracke KR, Joos GF, Maes T and Brusselle GG. Characterization and quantification of innate lymphoid cell subsets in human lung. Plos One. 2016; 11(1):e0145961. (* equal contribution). IF: 3.057, ranking in multidisciplinary sciences: 9/57.

ABSTRACT

Background: Innate lymphoid cells (ILC) are a new family of innate immune cells that have emerged as important regulators of tissue homeostasis and inflammation. However, limited data are available concerning the relative abundance and characteristics of ILC in the human lung.

Methods: The aim of this study was to characterize and enumerate the different ILC subsets in human lung by multi-color flow cytometry.

Results: Within the $CD45^+$ Lin^- $CD127^+$ pulmonary ILC population, we identified group 1 (ILC1), group 2 (ILC2) and group 3 (ILC3) innate lymphoid cells using specific surface markers (i.e. IL12Rβ2, CRTH2 and CD117 respectively) and key transcription factors (i.e. T-bet, GATA-3 and RORγT respectively). Based on the presence of NKp44, ILC3 were further subdivided in natural cytotoxicity receptor $(NCR)^+$ and NCR^- ILC3. In addition, we demonstrated the production of signature cytokines IFN-γ, IL-5, IL-17A, IL-22 and GM-CSF in the pulmonary ILC population. Interestingly, we observed a tendency to a higher frequency of NCR^- ILC3 in lungs of patients with chronic obstructive pulmonary disease (COPD) compared with controls.

Conclusions: We show that the three main ILC subsets are present in human lung. Importantly, the relatively abundance of ILC subsets tended to change in COPD patients in comparison to control individuals.

INTRODUCTION

Innate lymphoid cells (ILC) are a newly characterized heterogeneous family of the innate immune system, which have emerged as important regulators of tissue homeostasis, immunity and inflammation. ILC are morphologically similar to their counterpart of the adaptive immune system, T- and B-cells. Although they lack specific rearranged antigen receptors, which is a hallmark of the adaptive immune system, these innate immune cells can produce an array of cytokines in response to various danger signals and changes in homeostasis [199, 325]. Analogous with T-cells, ILC have been classified into three subsets – group 1 (ILC1), group 2 (ILC2) and group 3 (ILC3) innate lymphoid cells – depending on their phenotype, function and transcriptional regulation. The ILC1 subset includes both natural killer (NK) cells and non-toxic ILC1. These non-toxic ILC1 are characterized by the expression of the transcription factor T-bet and production of IFN-γ in response to interleukin (IL)-12. ILC2 depend on the transcription factor GATA-3 for their function and development and produce the type 2 cytokines, IL-5 and IL-13, in response to IL-25 and IL-33. Finally, the ILC3 subset is divided into natural cytotoxicity receptor (NCR, i.e. NKp44, NKp46 and NKp30)$^{+}$ ILC3 and NCR^{-} ILC3. The latter group is a heterogeneous population which also encompasses lymphoid tissue inducers (LTi) cells. These ILC3 express the transcription factor RORγT and are capable to produce IL-17A, IL-22 or granulocyte-macrophage colony-stimulating factor (GM-CSF) in response to IL-23 and IL-1β [198, 326].

Over the last few years, human ILC subsets have been studied in several tissues, including the skin and intestines [326]. To characterize these ILC, several surface markers have been proposed based on studies in the gut [198]. To our knowledge, research into ILC in the human respiratory system is currently limited to ILC2 [264, 327-331]. Recently, one study showed the presence of ILC subsets in human lung tissue in the context of lung cancer [332]. However, the relative abundance of ILC1, NCR^{+} ILC3 and NCR^{-} ILC3 in human lung tissue under inflammatory conditions such as chronic obstructive pulmonary disease (COPD) has not yet been characterized. COPD is a chronic inflammatory lung disease that is associated with the development of emphysema and lymphoid follicles. It has been shown that besides the adaptive immune system also innate immune cells substantially contribute to the pathogenesis of COPD [53].

In this manuscript, we identified and quantified non-toxic ILC1, ILC2, NCR^{+} ILC3 and NCR^{-} ILC3 subsets in human lung by flow cytometry based on several phenotypical markers and signature transcription factors. Further, we examined the expression of specific cytokines in the pulmonary ILC population. Finally, we compared the relative abundance of the ILC subsets in control subjects and patients with COPD.

MATERIAL AND METHODS

Lung tissue

Lung tissue was obtained from patients who underwent a surgical lung resection at Ghent University Hospital for solitary pulmonary tumors. Tissue was collected by a pathologist at maximum distance from the lung lesion, and showed no signs of retro-obstructive pneumonia or tumor invasion. Sixteen subjects were enrolled in our study and classified into 2 groups: 5 control subjects and 11 patients with COPD stage I or II. COPD diagnosis and severity was defined using pre-operative spirometry according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification: all control subjects had a post-bronchodilator ratio of forced expiratory volume in 1 second (FEV1) to forced vital capacity (FVC) above 70%, whereas all COPD patients had a FEV1/FVC ratio below 70%. Patients were diagnosed with COPD GOLD I when FEV1≥ 80%, patients with COPD GOLD II had a FEV1 between 50 and 80% predicted [333]. Written informed consents were obtained from all subjects, according to the protocol approved by the medical ethical committee of Ghent University Hospital. Patient characteristics are shown in Table 1.

Single cell suspensions from lung tissue

Lung resection specimens were processed as described previously to obtain single cell suspensions [334]. In brief, lung tissue was rinsed with physiological water (0.9 % NaCl) to remove residual blood. The lung tissue was cut into fine pieces and digested for 45 minutes at 37°C in digestion medium (Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine, 0.05 mM 2-mercaptomethanol (all Invitrogen), 100 U/ml penicillin, 100 mg/ml streptomycin (Sigma-Aldrich), 1 mg/ml collagenase type 2 (Worthington Biochemical), and 0.02 mg/ml DNase I (grade II from bovine pancreas; Boehringer Ingelheim)). Cells were resuspended in 10 mM ethylenediaminetetraacetic acid (EDTA) for 5 minutes at room temperature on a shaker. Next, cells were filtered through a 40-µm cell strainer and mononuclear cells were isolated with Ficoll-PaqueTM plus (GE Healthcare). Finally, cells were subjected to red blood cell lysis **(Fig. S1)**.

Table 1: Subject Characteristics

Characteristics	Controls n = 5	COPD n = 11
Gender (male/female)	3/2	10/1
Age, years [1]	60 ± 11	67 ± 9
BMI [1, 2]	25 ± 5	26 ± 5
Smoking history, Pack Years [1]	8 ± 13	36 ± 11
Smoking Status (never /current/ex smoker)	3/2/0	0/5/6
COPD GOLD Stage	na[3]	I / II
FEV1 % predicted, post-bronchodilator [1, 4]	99 ± 9	89 ± 16
FVC % predicted, post-bronchodilator [1, 5]	107 ± 12	102 ± 18
FEV1/FVC, post-bronchodilator [1, 4, 5]	77 ± 5	64 ± 5

[1] Data are expressed as mean ± standard deviation.
[2] BMI: body mass index.
[3] na: not applicable.
[4] FEV1: forced expiratory volume in 1 s.
[5] FVC: forced vital capacity.

Flow cytometry

Single cell suspensions were pre-incubated with human IgG to reduce nonspecific binding. For surface staining, the following human monoclonal antibodies were used: fluorescein isothiocyanate (FITC)-conjugated anti-CD45 (HI30), peridinin chlorophyll protein-cyanine 5.5 (PerCP)-conjugated anti-CD3 (OKT3), anti-CD19 (HIB19), anti-CD11c (3.9), anti-CD11b (M1/70), allophycocyanin (APC)-conjugated anti-NKp44 (P44-8), PE/indotricarbocyanine (Cy7)-conjugated anti-CD117 (104D2), brilliant violet 421TM-conjugated anti-CD127 (A019D5), brilliant violet 605TM-conjugated anti-CD56 (HCD56) (all from Biolegend); phycoerythrin (PE)-conjugated anti-IL12Rβ2 (305719; R&D systems), biotinylated anti-CRTH2 (BM19; Miltenyi Biotec) in combination with streptavidin (SAV)-APC or SAV-APC-Cy7 (BD Biosciences). For cytoplasmatic cytokine staining, cells were simulated for 15 hours with phorbol myristate acetate (PMA) and ionomycin, supplemented with brefeldin A and monensin (eBioscience) at 37°C. The

intracellular fixation and permeabilization buffer set (eBioscience) was used for fixation and cell permeabilization. The following antibodies were used: PE-conjugated anti-IL-17A (eBio64CAP17), anti-IFN-γ (4S.B3), anti-IL-22 (22URTI), anti-IL-5 (TRFK5), anti-GM-CSF (GM2F3), and isotype-matched control antibodies (all eBioscience). For nuclear staining, Foxp3/transcription factor staining buffer (eBioscience) set was used in combination with the following antibodies: PE-conjugated anti-T-bet (eBio4B10), anti-GATA-3 (TWAJ), anti-RORγT (AFKJS-9), and isotype-matched control antibodies (all eBioscience).

Data acquisition and analysis

Data acquisition was performed on a LSRFortessa running DiVa software (BD Biosciences). Cell subsets were analyzed using FlowJo Software. Statistical analysis was performed with SPSS, version 22.0 (SPSS, Chicago, USA). A non-parametric test (Mann-Whitney-U) was used to compare the different ILC subsets in the control versus COPD group, according to the standard statistical criteria. P-values < 0.05 were considered as significant. Patient characteristics are expressed as the mean ± standard deviation (SD). Flow cytometric data are expressed as the mean ± standard error of the mean (SEM).

RESULTS

Characterization of innate lymphoid cell subsets in human lung

In humans, the presence of ILC subsets has been studied in blood, gut and skin. In order to characterize ILC subsets in human lung tissue, we analyzed surgical lung resection specimens **(n = 16; patient characteristics are shown in table 1)**. Single cell suspensions were stained to identify the different ILC subsets. Fluorescence-minus-one controls were used to set flow cytometric gates **(Fig. S2)**. ILC were characterized as $CD45^+$ cells, a marker that is present on all hematopoietic cells. In addition, ILC lack the expression of specific lineage (Lin) markers (i.e. CD3 (T-cells), CD19 (B-cells), CD11b (neutrophils/eosinophils) and CD11c (dendritic cells/macrophages)), but express CD127 (IL-7Rα), the receptor for IL-7. We further refer to these $CD45^+$ Lin- $CD127^+$ cell population as the pulmonary ILC population **(Fig. 1A)**.

To make a distinction between the different ILC subsets within the pulmonary ILC population, we used specific surface markers. Since NK cells could contaminate the ILC1 subset, we used the CD56 marker to exclude NK cells and characterized the non-toxic ILC1 subset as $CD56^-$ $IL12R\beta2^+$ pulmonary ILC **(Fig. 1B)**. The ILC2 subset was identified as pulmonary ILC that express the chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRTH2), in analogy with ILC2 that previously were detected in the upper airways **(Fig. 1C)**. Both ILC3 subsets, NCR^+ ILC3 and NCR^- ILC3, were gated as $CD117^+$ (c-kit) pulmonary ILC and a further distinction was made by NKp44, a natural cytotoxicity receptor **(Fig. 1D)**. Using these staining combinations, we were able to discriminate between the three ILC subsets in human lung tissue. Moreover, we demonstrated that our staining combination was specific, since the surface markers that were used to characterize a specific ILC subset, were not expressed on other ILC subsets **(Fig. S3)**. Interestingly, inclusion of extra lineage markers (i.e. CD1a, CD14, CD34, CD123, TCRαβ, TCRγδ, BDCA2 and FcεR1) resulted in identical percentages of the different ILC subsets **(Fig. S4)**.

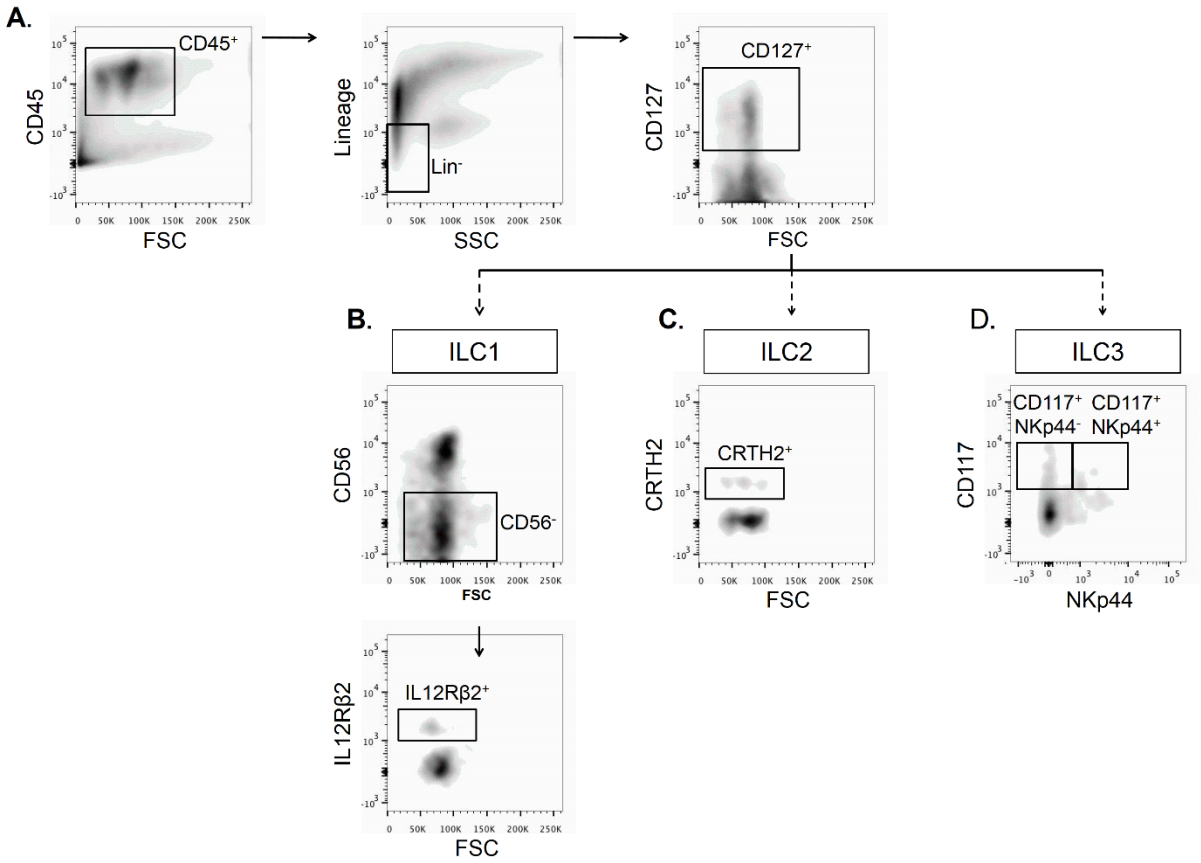


Figure 1: Characterization of innate lymphoid cell subsets in human lung. ILC subsets were identified by flow cytometry on single cell suspensions of digested human lung (n = 16). Fluorescence-minus-one controls were used to set gates (Fig. S2). **A,** The pulmonary ILC population was characterized as CD45$^+$, Lin$^-$ (i.e. CD3, CD19, CD11c, CD11b) and CD127$^+$ cells. Subsets were further defined based on specific surface markers. **B,** Non-toxic ILC1 were further gated as CD56$^-$, IL12Rβ2$^+$ cells. **C,** The ILC2 subset expresses CRTH2. **D,** NCR$^+$ ILC3 were CD117$^+$, NKp44$^+$, whereas NCR$^-$ ILC3 were characterized as CD117$^+$, NKp44$^-$ cells.

Intracellular staining of transcription factors in pulmonary ILC subsets

Besides characterization based on surface markers, also specific transcription factors can be used to characterize ILC subsets. For that purpose, we assessed the expression of key transcription factors within the different ILC subsets using intracellular flow cytometry. T-bet expression was detectable within the non-toxic ILC1 subset and – interestingly – also in the ILC3 subset, but not in the ILC2 subset **(Fig. 2A)**. The transcription factor GATA-3 was clearly expressed in the ILC2 subset, whereas no to limited GATA-3 levels were found in the ILC1 and ILC3 subsets **(Fig. 2B)**. Finally, specific expression of RORγT was demonstrated in the ILC3 subset; which contrasts with the ILC1 and ILC2 subsets that were both negative for the transcription factor RORγT **(Fig. 2C)**.

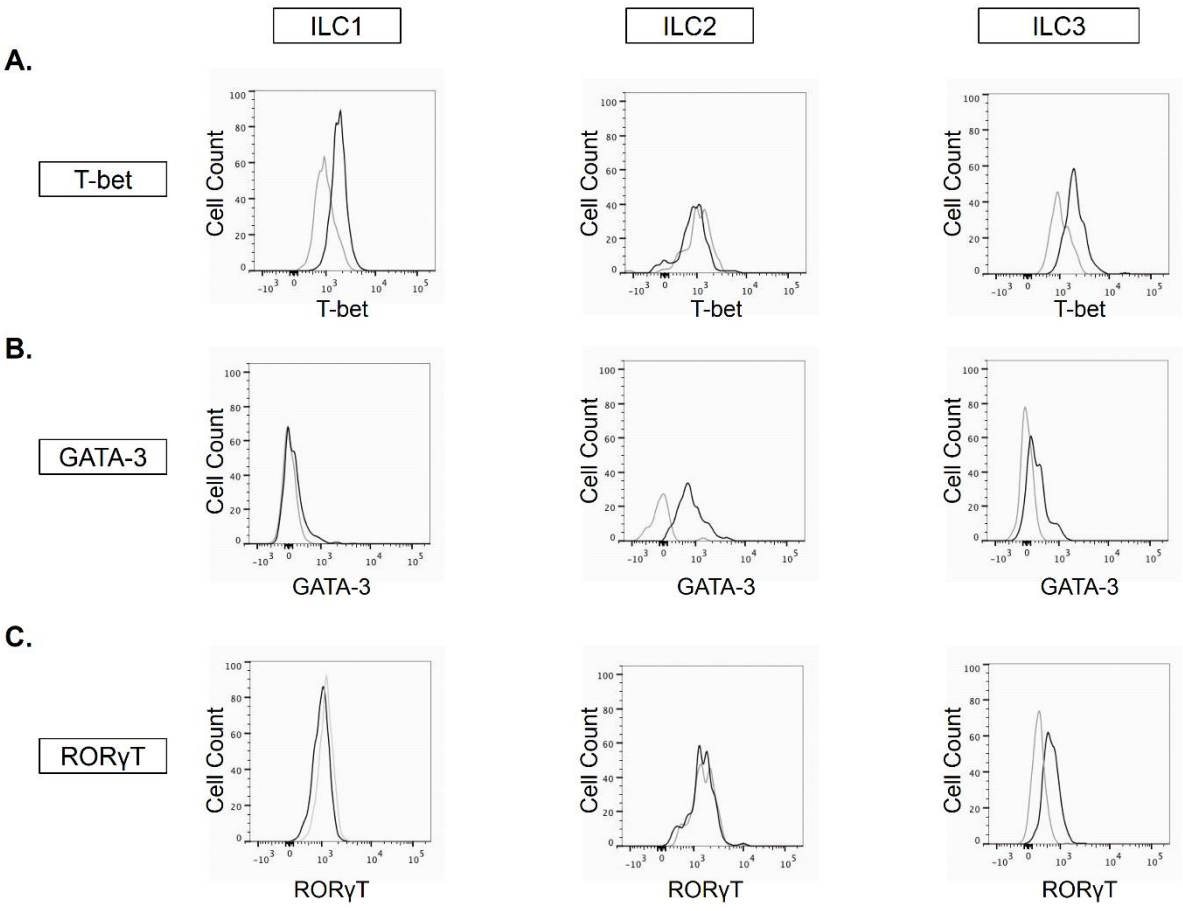


Figure 2: Intracellular staining of transcription factors in pulmonary ILC subsets. The developmental transcription factors were determined in the specific ILC subsets on single cell suspensions of digested human lung. **A,** Expression of T-bet (black line) versus isotype control (grey line) in the ILC1 ($CD45^+$, Lin^- $CD127^+$, $CD56^-$, $CRTH2^-$, $CD117^-$), ILC2 ($CD45^+$, Lin^-, $CD127^+$, $CRTH2^+$), ILC3 ($CD45^+$, Lin^-, $CD127^+$, $CD117^+$) population. **B,** GATA-3 expression (black line) versus isotype control (grey line) in the different ILC subsets. **C,** Expression of RORγT (black line) versus isotype control (grey line) in ILC1, ILC2 and ILC3 population.

Intracellular cytokine production in the pulmonary ILC population

To assess signature cytokines via intracellular flow cytometry, we stimulated lung single cell suspensions for 15 hours with PMA/ionomycin (+ protein transport inhibitors). Due to the scarcity of individual ILC subsets in human lung, signature cytokines where examined in the total pulmonary ILC population (i.e. $CD45^+$ Lin^- $CD127^+$ cells). Additionally, since NK cells are able to produce type 1 cytokines, CD56 was used as an extra exclusion marker during the analyses of IFN-γ. We observed production of IFN-γ, indicative for ILC1 in the human lung tissue **(Fig. 3A)**. Similarly, we could identify type 2 cytokine production, specifically IL-5 expression, which suggested the presence of pulmonary ILC2 in the human lung tissue **(Fig. 3B)**. Finally, also cytokines produced by ILC3 such as IL-17A **(Fig. 3C)**, IL-22 **(Fig. 3D)** and GM-CSF **(Fig. 3E)** were seen within the pulmonary ILC population. **Fig. 3F** shows the frequency of cytokine positive cells

in the ILC population. Of note, unstimulated pulmonary ILC had no to limited basal cytokine expression (data not shown).

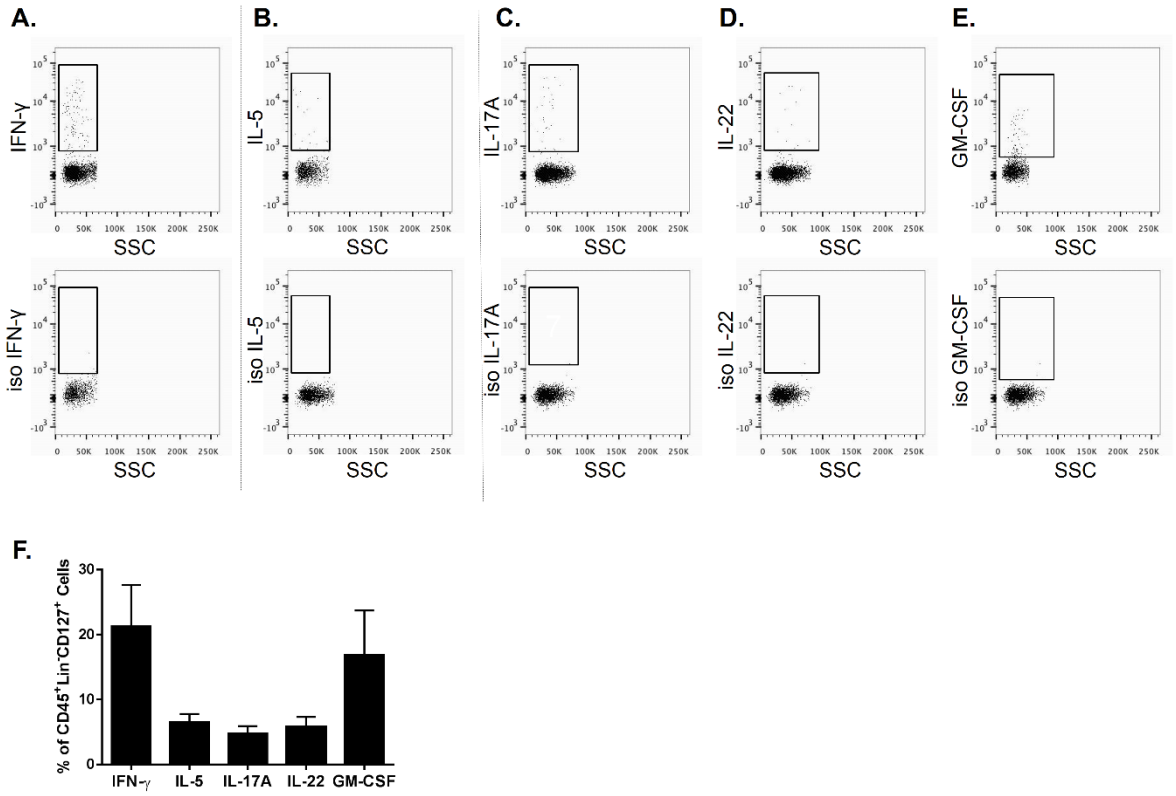


Figure 3: Intracellular cytokine production in the pulmonary ILC population. Several signature cytokines in pulmonary ILC (gated as $CD45^+$, Lin^- $CD127^+$ cells) were determined on single cell suspensions of digested human lung (n = 8). Since NK cells could contaminate the non-toxic ILC1 subset, $CD56^+$ cells were excluded to investigate the IFN-γ production. For the production of these cytokines, lung cells were first stimulated for 15 hours with PMA/ionomycin (+ transport inhibitors). **A,** IFN-γ production in ILC. **B,** Production of IL-5 in the pulmonary ILC population. **C,** IL-17 production in ILC. **D,** ILC production of IL-22. **E,** Production of GM-CSF in the ILC population. The bottom panels represent the isotype controls of the specific cytokine staining. **F,** Frequency of IFN-γ, IL-5, IL-17A, IL-22 and GM-CSF positive cells within the ILC (CD45+, Lin- CD127+) population (n = 8, mean ± SEM).

ILC subsets in control subjects versus patients with COPD

Using the above described surface markers, we quantified the percentages of ILC1, ILC2, NCR^+ ILC3 and NCR^- ILC3 subsets in lung single cell suspensions. Since the relative abundance of pulmonary ILC subsets could be altered in diseases such as COPD, we investigated the frequency of the different ILC subsets in control subjects (n=5) and in patients with COPD (n=11). Although there were no significant differences observed in the relative abundance of the specific ILC subsets between the control and COPD group (probably due to low patient numbers) **(Fig. 4A)**, some interesting findings were found. In the control group, both ILC2 and NCR^- ILC3 were the most abundant ILC subsets. Interestingly, in patients with COPD the distribution of ILC tended to shift to a greater presence of NCR^- ILC3 compared with the other ILC subsets **(Fig. 4B)**. Moreover, when

analysing cytokine positive ILC, we observed that IL-17A and IL-22 expressing ILC tended to increase in patients with COPD compared with controls, whereas IFN-γ and IL-5 expressing ILC were similar **(Fig. S5)**.

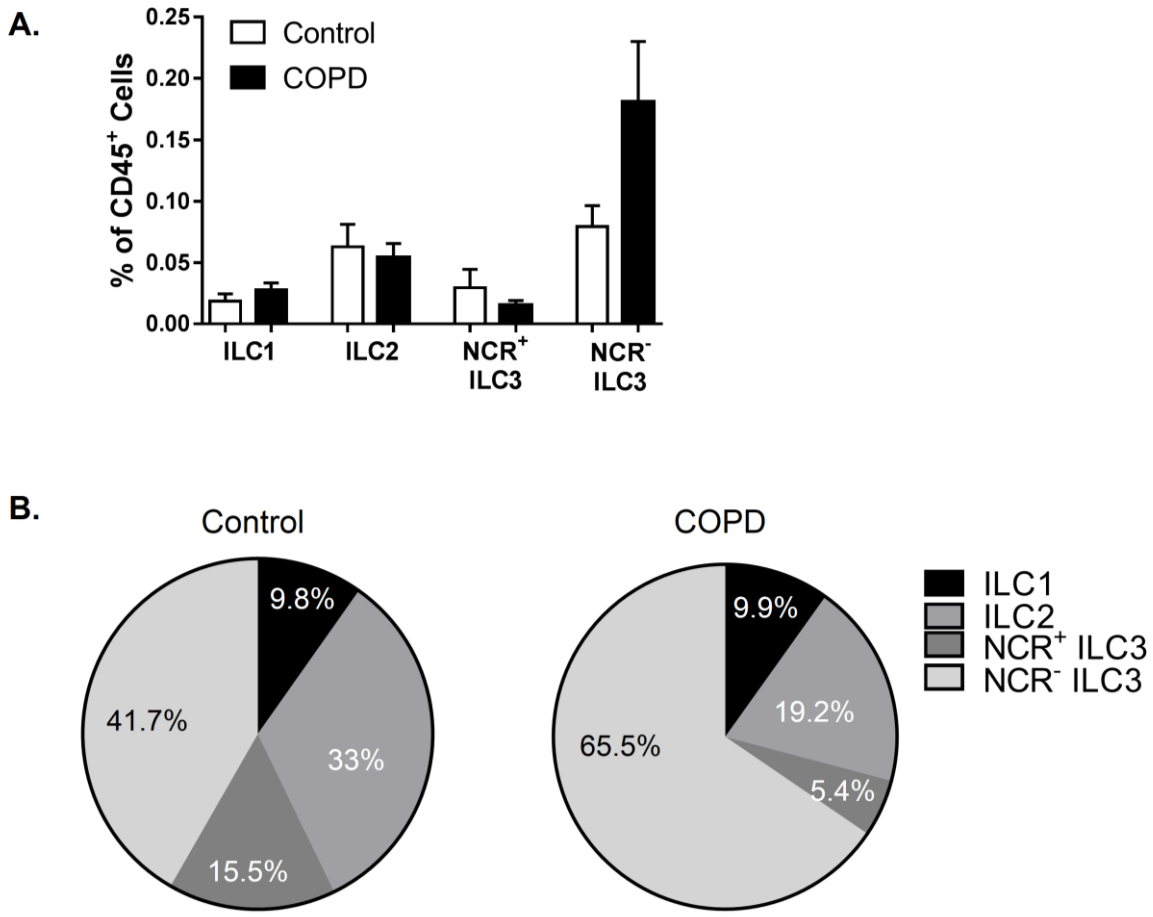


Figure 4: ILC subsets in control subjects versus patients with COPD. A, The frequency of ILC1 ($CD45^+$, Lin^-, $CD127^+$, $CD56^-$, $IL12R\beta2^+$), ILC2 ($CD45^+$, Lin^-, $CD127^+$, $CRTH2^+$), NCR^+ ILC3 ($CD45^+$, Lin^-, $CD127^+$, $CD117^+$, $NKp44^+$) and NCR^- ILC3 ($CD45^+$, Lin^-, $CD127^+$, $CD117^+$, $NKp44^-$) in digested human lung from control (n = 5) and COPD patients (n = 11) was determined by flow cytometry. ILC numbers were expressed as percentages (%) of the CD45+ population (mean ± SEM). **B,** Pie chart of the relative abundance of ILC1, ILC2, NCR^+ ILC3 and NCR^- ILC3 subsets in control subjects and patients with COPD.

DISCUSSION

We demonstrate the presence of all ILC subsets in human lung **(Fig. 5)**. Using specific surface markers and key transcription factors, we characterized the ILC1, ILC2, NCR$^+$ ILC3 and NCR$^-$ ILC3 subsets in human lung single cell suspensions by multi-color flow cytometry. In addition, we assessed the production of signature cytokines in the pulmonary ILC population. Furthermore, our data suggest that the frequency of NCR$^-$ ILC3 tended to increase in patients with COPD.

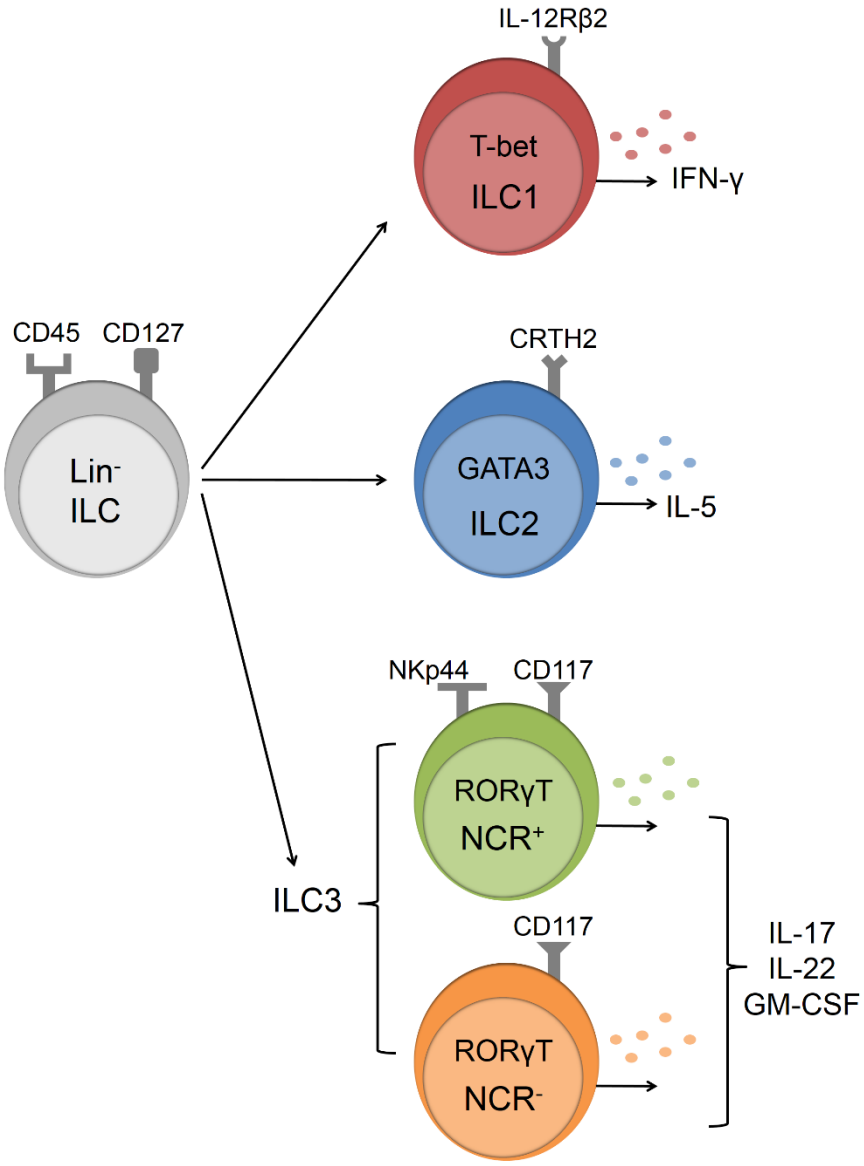


Fig. 5. Overview of innate lymphoid cell subsets in human lung tissue. The presence of CD45$^+$, Lin$^-$ (i.e. CD3, CD19, CD11c, CD11b) and CD127$^+$ ILC in pulmonary tissue was demonstrated. These ILC were further subdivided in a CD56$^-$ IL12Rβ2$^+$ ILC1 subset, CRTH2$^+$ ILC2 subset, CD117$^+$ NKp44$^+$ (NCR$^+$) ILC3 subset and CD117$^+$ NKp44$^-$ (NCR$^-$) ILC3 subset. Further, expression of signature transcription factors (i.e. T-bet, GATA-3 and RORγT) within the specific ILC subset and cytokine production (i.e. IFN-γ, IL-5, IL-17A, IL-22 and GM-CSF) within the pulmonary ILC population was demonstrated.

To characterize ILC subsets in human tissues, specific surface markers have been proposed in the literature. In gut mucosal tissue, a higher fraction of the IL12Rβ2 transcript could be detected in the ILC1 population [335]. Using this specific marker, as well as CD56 to exclude contaminating NK cells [198, 336], we identified lung ILC1 as $CD45^+$ Lin^- $CD127^+$ $CD56^-$ $IL12R\beta2^+$ cells. However, it should be noted that some controversy still exists in the characterization of the (human and mouse) ILC1 subset. At least in the gut, an additional intraepithelial $CD127^{low}$ $CD103^+$ ILC1 subset has been discovered, that would be the equivalent of cytotoxic $CD8^+$ T-cells [326]. Recently, such a $CD127^{low}$ $CD103^+$ ILC1 subset was also found in human lung tissue [332]. Compatible with research findings in the gut and nasal polyps [331], we identified pulmonary ILC2 as $CRTH2^+$ cells within the pulmonary ILC population. ILC3 subsets in the gut and skin were distinguished based on NCR (NKp44) [337, 338]. Accordingly, in human lung specimens, we show the presence of both NCR^+ ILC3 and NCR^- ILC3 within the $CD117^+$ pulmonary ILC. One should however be aware that the NCR^- ILC3 subset remains a heterogeneous population that also contains LTi-cells.

In addition to the characterization based on surface markers, the expression of developmental transcription factors is an important feature to identify ILC subsets. We demonstrated a clear expression of GATA-3 in the ILC2 subset and RORγT in the ILC3 subset, which suggests that our staining based on surface markers adequately discriminates between the ILC subsets. Of interest, besides T-bet expression in the ILC1 subset, we also demonstrated a high T-bet expression in the ILC3 subset. It was previously shown that, upon stimulation with IL-12, intestinal NCR^- ILC3 could lose their RORγT expression and upregulate T-bet, suggesting that ILC3 can differentiate into ILC1 [335, 339]. Our observed T-bet signal in the ILC3 subset could therefore indicate a plasticity of human pulmonary ILC3, although this requires further investigation.

Upon stimulation, ILC are able to produce several effector cytokines. We observed expression of IFN-γ, IL-5, IL-17A and IL-22 in the pulmonary ILC population, indicative for the presence of activated ILC1, ILC2, NCR^+ ILC3 and NCR^- ILC3. In addition, we also observed GM-CSF production which would be interesting to explore further, since $ROR\gamma T^+$ ILC3 were able to produce GM-CSF in the mouse intestine which contributed to T-cell homeostasis [220, 340]. However, it should be emphasized that the cytokine expression was investigated in the total pulmonary ILC population. Assessment of cytokine production by the specific pulmonary ILC subsets could provide additional functional insights. Furthermore, it would be worthwhile to investigate the production of amphiregulin by human pulmonary ILC2 in future experiments. At least in mice, it has been shown that besides type 2 cytokines, ILC2 can produce amphiregulin, an epidermal growth factor that has been implicated in wound healing and tissue (lung) remodelling [327].

Depending on the tissue, the composition of human ILC subsets can differ [326]. In healthy skin, for instance, higher numbers of ILC2 and NCR$^-$ ILC3 were found [337], whereas NCR$^+$ ILC3 are the most abundant ILC subset in the gut [341]. The number of ILC subsets in our control subjects is relatively low, but is in line with the number of ILC2 that was previously observed in non-inflamed nose tissue [331]. Further, it has been shown that the relative abundance of the ILC subsets can depend on the disease state, such as in Crohn's disease, psoriasis and chronic rhinosinusitis [331, 335, 337]. Although there were no statistical differences between the control and COPD group, a trend to a higher relative abundance of NCR$^-$ ILC3 could be observed in patients with COPD. It should however be noted that a small number of patients was investigated, and that our findings need to be confirmed in a larger study population.

In COPD patients, a higher number of lymphoid follicles was found compared to controls that correlated with disease severity [342, 343]. Importantly, IL-17A and IL-22, which are produced by NCR$^-$ ILC3, are crucial in the formation of lymphoid follicles [344, 345]. It would therefore be interesting to investigate the role of the increased NCR$^-$ ILC3 subset in lymphoid follicle formation in patients with COPD. Furthermore, as lymphoid follicles are also observed in other chronic pulmonary diseases (i.e. pulmonary fibrosis, bronchiectasis, follicular bronchiolitis, lymphoid interstitial pneumonia) [343], it would be worthwhile to also examine the relative abundance of NCR$^-$ ILC3 in these disease settings. Besides being involved in the formation of lymphoid follicles, NCR$^-$ ILC3 could also contribute to the persistent inflammation in COPD patients by pro-inflammatory cytokine production. Alternatively, it could be that the accumulation of NCR$^-$ ILC3 in COPD is associated with host protective immunity, in response to bacterial respiratory tract infections which occur frequently in patients with COPD [343]. Further functional studies that address the role of NCR$^-$ ILC3 in COPD are therefore warranted. Very little is known about the (protective and pathological) role of ILC3 in lung homeostasis as well as in the context of disease. Recently, NCR$^-$ ILC3 were found to accumulate in human non-small cell lung cancer tissue, where they might contribute to the formation of protective tumour-associated tertiary lymphoid structures [332].

Until now, studies in the respiratory system have mainly focused on the ILC2 subset. Although several animal studies have described an important role for ILC2 in helminth infections [214], allergic airway inflammation [158, 234, 241, 346] and airway hyperresponsiveness [242, 312], the function of ILC2 in the human lung remains incompletely studied. To date, increased numbers of ILC2 in nasal polyps from patients with chronic rhinosinusitis have been demonstrated [330, 331], suggesting a role for ILC2 in eosinophilic inflammation in the upper airways. In addition, genes discovered in genome-wide association studies of asthma (RORα, IL-13, IL-33, IL-1RL1;

which are all related to ILC2) suggest a key role for ILC2 in asthma [16]. We recently hypothesized that ILC2 could have an important role in non-allergic eosinophilic airway inflammation [270]. In support of this, research in an experimental model of asthma has shown that ILC2 are highly corticosteroid resistant [271], which could explain why severe eosinophilic asthmatics are relatively corticosteroid resistant [270].

In summary, based on expression of surface markers and key transcription factors, we demonstrated that ILC1, ILC2, NCR^+ ILC3 and NCR^- ILC3 subsets are present in the human lung. In addition, pulmonary ILC were able to produce signature cytokines upon stimulation. Of interest, we showed that pulmonary NCR^- ILC3 tended to accumulate in the lung of COPD patients, although this should be confirmed in a larger study population. The functional role of ILC subsets in lung homeostasis and pulmonary diseases however remains to be fully elucidated. Further research on the function of ILC subsets is therefore needed to address whether ILC are possible targets for new therapeutics in (chronic) pulmonary diseases.

SUPPLEMENTAL FIGURES

Fig. S1: Process of making single cell suspensions of lung resection specimen. Lung tissue was obtained from patients who underwent a surgical lung resection. Tissue was cut into fine pieces and digested for 45 minutes at 37°C in digestion medium supplemented with collagenase type 2 and DNase I. Next, EDTA was added to stop the digestion and the lung cells were filtered through a 40-µm cell strainer. Pulmonary mononuclear cells were isolated with Ficoll-PaqueTM plus. Finally, cells were subjected to red blood cell (RBC) lysis and stained for flow cytometry.

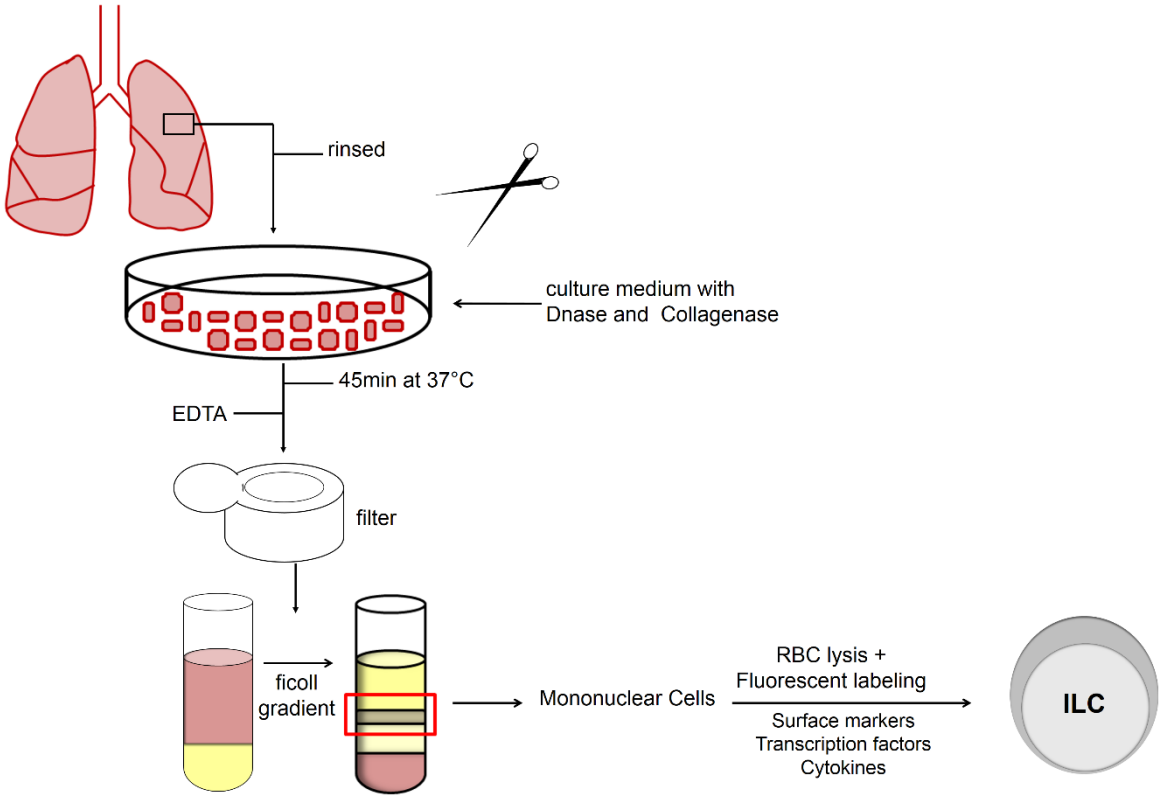


Fig. S2: Fluorescence minus one (FMO) controls to set up an adequate ILC gating strategy. FMO controls were used on single cell suspensions of digested human lung and analyzed by flow cytometry. FMO controls contain every stain in the ILC panel except for that specific fluorochrome that was investigated. This figure shows how the gate for the specific ILC subsets **(Fig. 1)** was set as compared to the different FMO controls.

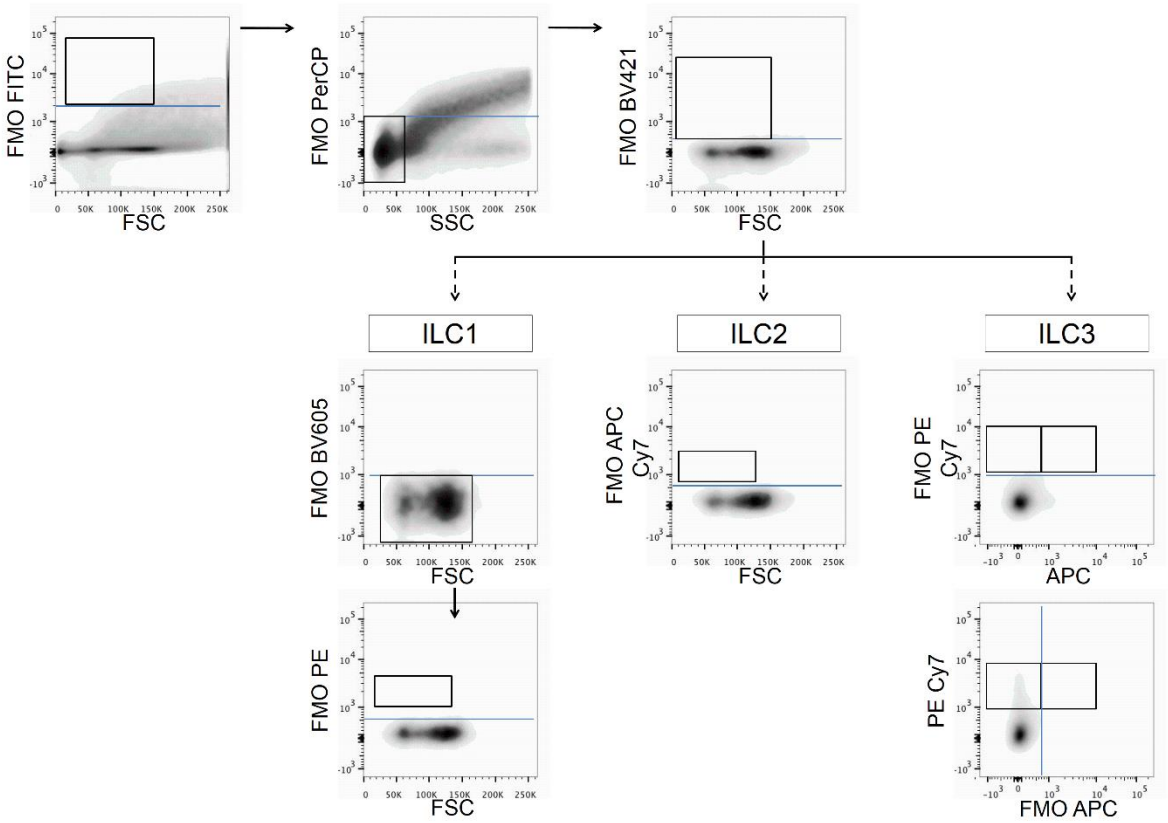


Fig. S3: Specificity of ILC staining combination. A, Expression of CRTH2, CD117 in the pulmonary ILC1 population. **B,** Analyses of the surface markers IL12Rβ2, CD117 in the ILC2 subset. **C,** Expression of IL12Rβ2, CRTH2 in the pulmonary ILC3 subset.

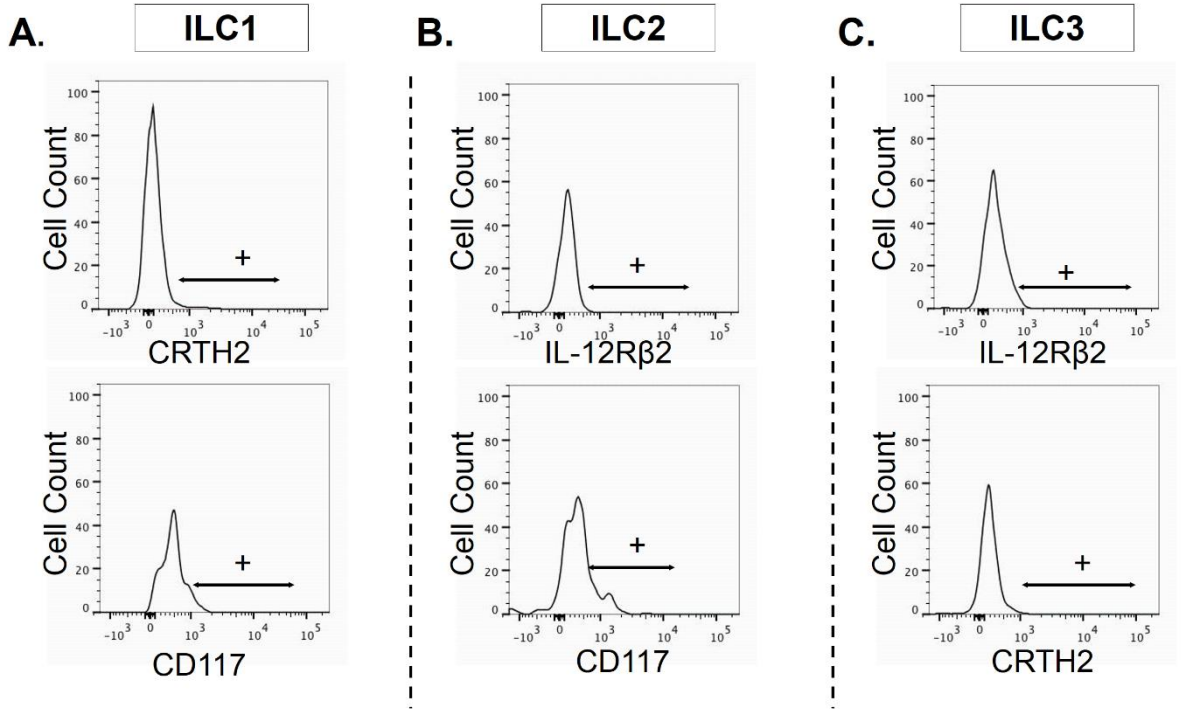


Fig. S4: Inclusion of extra lineage markers in the ILC cocktail. Inclusion of additional lineage markers in the ILC cocktail was assessed by flow cytometry on single cell suspensions of digested human lung. Frequencies of ILC1, ILC2 and ILC3 in digested human lungs using our 'classical' lineage mix (i.e. CD3, CD19, CD11c, CD11b) (filled symbols) and the lineage mix with extra markers (i.e. CD3, CD19, CD11c, CD11b, CD1a, CD14, CD34, CD123, TCRαβ, TCRγδ, BDCA2 and FcεR1) (open symbols) are shown. n=3.

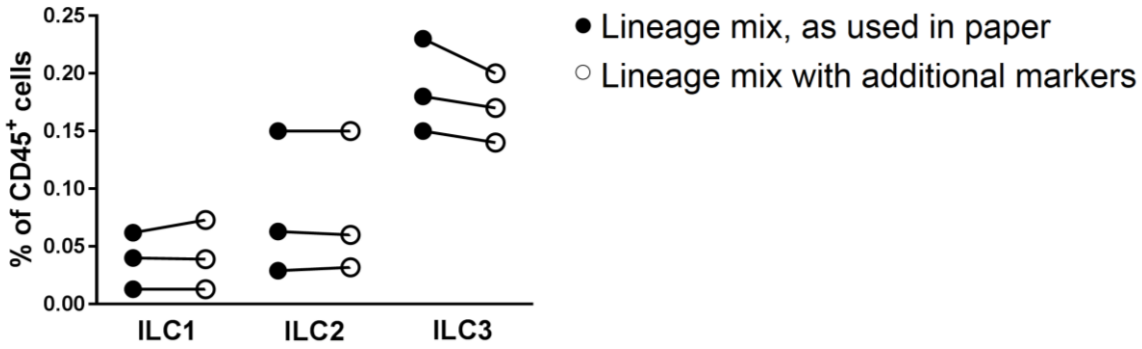
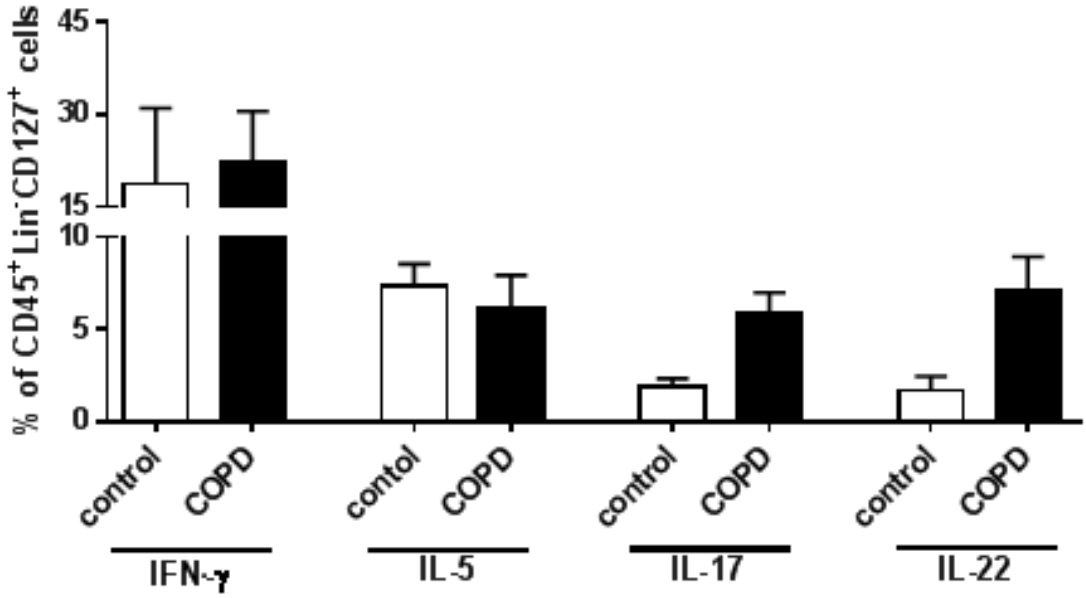


Fig. S5: Frequency of cytokine positive ILC in control subjects and patients with COPD. Frequency of IFN-γ, IL-5, IL-17A, IL-22 positive ILC (gated as $CD45^+$, Lin^-, $CD127^+$ cells) in digested human lung of control subjects (n=2) and COPD patients (n=6) was determined by intracellular flow cytometry staining (mean ± SEM).



CHAPTER 11: DISCUSSION AND FUTURE PERSPECTIVES

11.1 Diesel exhaust particles aggravate allergic airway inflammation

Exposure to air pollution is associated with many health problems, ranging from respiratory diseases, such as asthma, COPD and lung cancer, to cardiovascular illness and all-cause mortality [60]. In the lung, exposure to DEPs causes acute inflammation and augments allergen-induced immune responses. To study the aggravating effects of DEPs on allergic airway inflammation, we developed a murine model wherein a clinical relevant allergen, i.e. HDM, was administered simultaneously with DEPs. Whereas exposure to sole DEPs or HDM elicited minimal responses, concomitant DEPs+HDM exposure greatly enhanced several features of allergic airway inflammation, characterized by an excessive inflammatory cell recruitment, mucus metaplasia, type 2 cytokine production, HDM-specific IgG1 and AHR. These observations were in line with other experimental models wherein the enhancing effect of DEPs on allergic airway inflammation was also present [96, 314]. Although we can conclude that we have developed a murine model wherein simultaneous exposure to DEPs and HDM aggravated the allergic inflammatory responses, the exposure of these environmental factors could also occur at different time points in real life. Therefore, it would be worthwhile to repeat the experiment where DEPs and HDM are administered separately and investigate if the effects of DEPs on allergic responses may vary. At least in an OVA-model it was suggested that allergic sensitization and/or challenge prior to DEPs led to exaggerated airway responses [347-349]. In contrast, when DEPs was administered prior OVA sensitization an attenuated OVA-induced airway inflammation was observed [350].

11.2 Diesel modulates airway epithelial responses

Airway epithelial cells have been identified as crucial regulators, as epithelial-derived cytokines and chemokines can recruit, activate and instruct both DCs and ILCs, promoting type 2 mediated immune responses [102]. In the first part of this dissertation, we investigated the role of the airway epithelium in the development of DEPs-induced inflammatory lung responses. More specifically, we were interested in the role of some epithelial-derived signaling pathways, i.e. chemerin and IL-33 signaling. As DCs have been recognized at the frontline of DEPs-induced immune responses [94], we investigated the role of the chemerin/ChemR23 signaling pathway, implicated in DC recruitment, during acute DEPs and combined DEPs+HDM exposure. Secondly, the role of the IL-33/ST2 signaling pathway, which contributes to the activation of both DCs and ILC2s, was investigated in DEPs-enhanced allergic airway inflammation. Our findings highlight a

complex regulation of the epithelial airway responses towards different inflammatory conditions or administration settings, i.e. prophylactic vs therapeutic.

11.2.1 Chemerin/ChemR23 signaling

First, we demonstrated that chemerin is increased in the lungs of mice that were exposed to acute DEPs and combined DEPs+HDM. This is in line with observations where increased chemerin was detected in the lungs of CS-exposed mice [120] and in serum of COPD patients compared to the control group [135]. Moreover, the cellular source of prochemerin was airway epithelial cells, confirming previous reports [120, 131].

Whereas the inflammatory lung responses were abrogated in ChemR23 KO mice upon acute DEPs exposure, ChemR23 KO mice that were exposed to combined DEPs+HDM had increased type 2 mediated immune responses compared to WT controls. Our data therefore indicate that depending on the environmental stimuli, the chemerin/ChemR23 axis exerts different biological functions. Interestingly, this was also observed in other models wherein the ChemR23 axis had pro-inflammatory actions in response to CS [120], while anti-inflammatory properties were present during allergic airway responses [121].

How exactly different environmental stimuli affect the chemerin/ChemR23 axis remains to be further investigated. In this context, it was demonstrated that chemerin variants with distinct pharmacological properties can be formed depending on which proteases are available in the environment. Serine proteases for instance can generate active chemerin fragments [129, 284], whereas mast cell chymase is associated with an anti-inflammatory chemerin fragment [285, 286]. Since DEP inhalation induces massive neutrophil accumulation [94], one can hypothesize that upon acute DEPs exposure serine proteases will induce active chemerin, leading to cell chemotaxis into the airways. In contrast, one can speculate that during the DEPs-enhanced allergic airway inflammation, mast cells will induce the formation of chemerin-154, which exerts anti-inflammatory actions [285], by for instance inhibiting macrophage activation [286]. To test this in our models of DEPs-induced inflammatory lung responses, additional experiments should be performed where either neutrophils or mast cells are depleted or protease inhibitors are administered. Moreover, discriminating the different chemerin variants that are formed during DEPs-induced airway inflammation will lead to additional insights into the complex biology of chemerin signaling.

Secondly, one can wonder whether the effects of chemerin are indirectly mediated through the release of other (epithelial) mediators. Our group has previously shown that monocytes and

monocyte-derived DCs are recruited in a CCL2/CCR2, and to a lesser extent CCL20/CCR6 dependent manner upon DEP inhalation [95]. As in DEPs-exposed ChemR23 KO mice lower CCL2 and CCL20 levels were observed, this could imply that the reduced inflammatory lung responses upon DEP inhalation were an indirect consequence of chemerin on epithelial CCL2 and CCL20 production. However, in DEPs+HDM-exposed ChemR23 mice, where an enhanced allergic airway inflammation was present, also impaired CCL2 levels were detected. The opposing role of chemerin can therefore not be solely held attributable to a feedback mechanism of chemerin on epithelial CCL2 or CCL20 production. Besides chemerin, resolvin E1 (RvE1) is identified as a second ligand of ChemR23 [134], that can exert anti-inflammatory properties, leading to the resolution of allergic airway inflammation [294]. RvE1 is enzymatically derived from omega-3 fatty acids. Interestingly, intake of these fatty acids have been associated with lower asthma prevalence [351]. However, since we demonstrated reduced RvE1 levels upon both acute DEPs and combined DEPs+HDM exposure, RvE1 cannot explain the opposing inflammatory responses that were observed in the ChemR23 KO mice in the two models. Of note, as the anti-inflammatory effects of chemerin were not restricted to ChemR23 expressing cells in the model of DEPs-enhanced allergic airway inflammation, an indirect of chemerin was further suspected. This is in line with other models wherein it was demonstrated that chemerin can exert anti-inflammatory properties via an indirect effect on a non-leukocyte cell population (i.e. airway epithelium and endothelium) [121, 136]. Further studies should however be conducted to discover which mechanisms and mediators are influenced by chemerin. One could speculate that upon DEPs+HDM exposure, chemerin exerts anti-inflammatory effects by modulating leukocyte trafficking and/or multiple pro-inflammatory cytokines and chemokines.

11.2.2 IL-33/ST2 signaling

Expression analyses revealed that the mRNA levels of IL-33, present in structural cells, and ST2, located in hematopoietic cells, are significantly elevated in the lungs of DEPs+HDM-exposed mice. This was accompanied by increased full length and mature IL-33 protein levels in response to combined DEPs+HDM exposure. Accordingly, increased IL-33 expression was demonstrated in allergen or CS-exposed mice [159-161, 190] and asthmatic or COPD patients, which correlated with disease severity [125, 150, 187, 188]. Moreover, IL-33 production further increased in asthmatic bronchial epithelium that was exposed to PM [185].

Administration of recombinant sST2 (r-sST2) to mice from the start of the exposure protocol, i.e. prophylactic setting, lowered the DEPs-enhanced allergic airway inflammation. When DEPs+HDM-exposed mice were treated with r-sST2, a decreased amount of BALF eosinophils,

total number and cytokine-expressing ILC2s and T_H2 cells could be observed compared to PBS-treated controls. These results indicate that IL-33/ST2 signaling has a prominent role during DEPs-enhanced allergic airway responses. Our observations were in line with other models where an attenuation of the HDM-induced inflammatory cell recruitment could be observed in ST2-, IL-33 KO mice or upon r-sST2 administration [159-163]. However, the contribution of the IL-33/ST2 axis in allergic airway inflammation has been a matter of debate since several years, as a contradiction between results was obtained with different exposure models and IL-33 disrupting agents [163, 352]. Indeed, the development of an allergic airway inflammation has also been shown to occur independently of the IL-33 axis. Using IL-33 KO mice for instance, absence of IL-33 did not affect the HDM-driven airway inflammation, with the exception of cytokine-producing ILC2s [165]. Of note, also in our model r-sST2 lowered the total number and cytokine expressing ILC2s in sole HDM-exposed mice.

In contrast to our BALF data however, prophylactic administration of r-sST2 had no significant effect on the inflammatory lung responses (i.e. lung eosinophilia and goblet cell metapalasia), type 2 cytokine production in the MLN, HDM-specific IgG1 levels and AHR. Considering that we only observed a local response towards r-sST2, the half-life of r-sST2 or our intranasal application route could be questioned. It should however be noted that in a murine model of SpID-induced asthma, intratracheal administration of r-sST2 only modestly affected the inflammatory lung responses [301]. Moreover, also an intraperitoneal injection of r-sST2 at the time of HDM sensitization, showed no apparent differences in the type 2 immune responses in the lymph nodes and amount of goblet cells [161]. One can hypothesize that in our DEPs+HDM model the activity of r-sST2 is too limited to affect the inflammatory immune responses in the MLN and lung, and therefore additional experiments using ST2- or IL-33 KO mice could provide better insights. Interestingly, it was recently demonstrated that IL-33 blockade through r-sST2 prevented asthma in an age-dependent manner, wherein especially neonatal mice were susceptible for the treatment [161]. Maybe the r-sST2 treatment would also be more effective in DEPs+HDM-exposed neonatal mice. Moreover, considering that HDM-treated ST2 KO mice had a diminished goblet cell metaplasia and AHR in the peripheral lung, while no differences were observed in central airways [160], it would be worthwhile to investigate whether distinct effects of ST2 signaling in peripheral versus central airways can also be observed in our model of DEPs-enhanced allergic airway inflammation.

As it was previously demonstrated that anti-IL-33 treatment could decrease a PM-induced asthma exacerbation [186], we were interested if r-sST2 could also be used as a therapeutic upon combined DEPs+HDM exposure. However, when r-sST2 was administrated only at the end of the exposure protocol, no differences in airway immune responses could be observed, indicative that

IL-33/ST2 signaling is more important during the onset of the DEPs-enhanced allergic airway inflammation. This was in line with de Kleer et al. where treatment with r-sST2 during the challenge phase had no effect on the HDM-induced allergic airway inflammation, in contrast to a decreased inflammatory cell recruitment when r-sST2 was administered during the sensitization phase [161]. For the moment, human studies with anti-IL-33 are ongoing [37, 168] and still have to prove their beneficial effect in asthmatic patients (please see 11.4).

11.3 Innate lymphoid cells in pollutant-aggravated airway diseases

ILCs are early regulators of tissue homeostasis immunity and inflammation. Although ILCs can exert important functions in pathogen control and tissue repair, dysregulation of ILC can lead to the development of inflammatory diseases, such as asthma and COPD [194]. In the second part of this dissertation, we investigated the contribution of ILCs in pollutant-aggravated airway diseases. First of all, considering that ILC2s have been implicated in the pathogenesis of allergic asthma, we investigated the contribution of ILC2s and their counterpart of the adaptive immune system, i.e. T_H2 cells, during the aggravating effects of DEPs on allergic airway inflammation. Secondly, as human data concerning the different ILC subsets in the respiratory system were lacking, we characterized and quantified the different ILC subsets in *ex vivo* lung samples of control and COPD patients. Although we demonstrated that the relative abundance of ILCs can alter during pollutant-aggravated airway diseases, this does not by definition highlight a functional importance of the ILCs.

11.3.1 Role of ILC2s in DEPs-enhanced allergic airway inflammation

In our murine model, concomitant exposure to DEPs+HDM greatly enhances the allergic airway inflammation, characterized by excessive inflammatory cell recruitment, mucus metaplasia, type 2 cytokine production, HDM-specific IgG1 and AHR. Interestingly, we observed that concomitant DEPs+HDM exposure increased the activity of both ILC2s and T_H2 cells. As GATA-3 is an important transcription factor for the development and function of both cell types [315], the effects of GATA-3 modulation were investigated. We demonstrated that a reduced GATA-3 expression led to a decreased activity of both ILC2s and T_H2 cells in the BALF of DEPs+HDM-exposed mice, which was accompanied by decreased type 2 immune responses. These results indicate that enhancing effects of DEPs on allergic airway inflammation are dependent on an adequate GATA-3-mediated regulation of ILC2s and/or T_H2. Moreover, the modest inflammation observed after sole HDM

exposure was also abrogated upon reduced GATA-3 expression, which was in line with earlier results where the allergic airway inflammation was inhibited in GATA-3 mutant mice [322]. Of note, as it was demonstrated that GATA-3 is required for the development of all non-cytotoxic ILC populations [353], one can wonder if also ILC1s or ILC3s are affected and contribute to the decreased inflammatory immune responses.

Further, we observed that the aggravating effects of DEPs on allergic airway inflammation were completely abrogated in RAG KO mice, whereas the allergic airway responses only tended to decrease when ILC2s were absent. These data indicate a prominent role for the adaptive immune system during DEPs-enhanced allergic airway inflammation, whereas ILC2s only marginally contributed. Our results were however in contradiction with earlier reports where ILC2s were able to mediate an allergic airway inflammation even in the absence of an adaptive immune system [157, 158, 241, 354]. It should however be noted that in these studies recombinant IL-33, *Alternaria e*xtract or papain was used to induce specific allergic features. Moreover, by using ILC2 deficient mice, it was proposed that ILC2s arise first and are required for mounting robust T_H2 cell responses [170, 245]. In accordance with our study, it was however recently demonstrated that exposure to HDM was also unable to promote an eosinophilic airway inflammation in RAG KO mice. Moreover, a specific contribution of T cells, but not B cells, was proposed for the induction of ILC2s, leading to a HDM-mediated airway inflammation [165]. In this regard, it would be interesting to investigate in our DEPs+HDM model the inflammatory responses in KO mice that specifically lack T or B cells. Furthermore, interaction between T cells and ILC2s which promote DEPs-enhanced allergic airway inflammation require further investigation. At least during a HDM-mediated airway inflammation, it was proposed that both epithelium-derived signals, i.e. IL-33, as T cell-derived mediators such as IL-2 were needed to induce ILC2 activation [165]. Importantly, also mechanistic studies should be conducted to investigate how different environmental stimuli modulate the contribution of ILC2s. These insights would be helpful to predict which asthmatic phenotype would respond to ILC2s-modulating therapies.

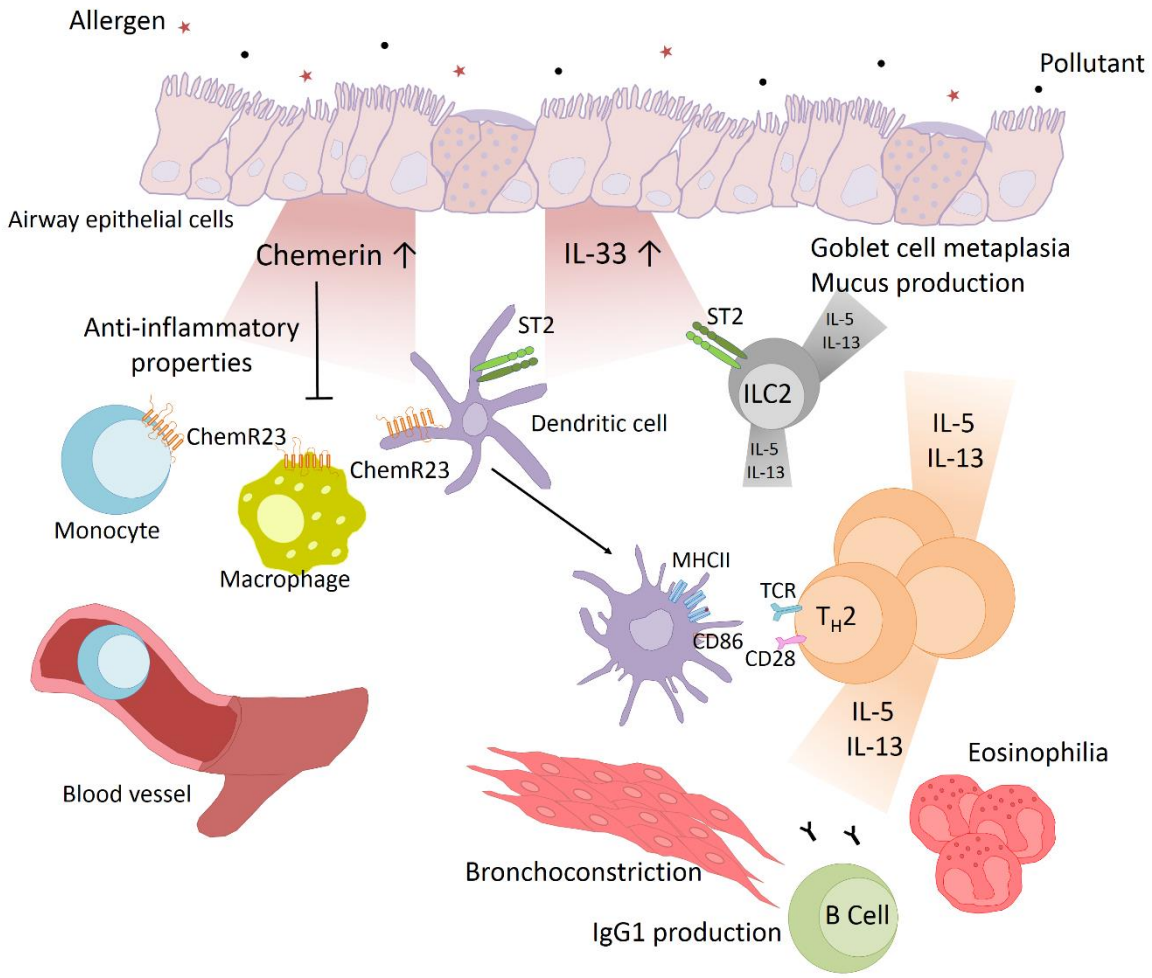


Figure 20: Mechanisms involved in the aggravating effects of DEPs on allergic airway inflammation. Combined DEPs+HDM exposure increases the expression of epithelial-derived chemerin and IL-33. Whereas chemerin exerts anti-inflammatory properties, IL-33 exerts a pro-inflammatory function, leading to the activation DCs and ILC2s. In contrast to the crucial role of T_H2 cells, ILC2 were demonstrated to be dispensable during the aggravating effects of DEPs on allergic airway inflammation, despite their type 2 cytokine production.

11.3.2 ILCs in human lung tissue

In human lung tissue, we started by investigating whether all ILC subsets were present. To characterize the different ILCs, first specific surface markers were investigated. Non cytotoxic lung ILC1s were distinguished based on the lack of CD56 (exclude conventional NK cells) and expression of IL12Rb2, which was found to be upregulated in human ILC1s derived from tonsils [335]. To date, characterization of ILC1s remains however challenging as they are a highly heterogeneous population, wherein besides $CD127^+$ ILC1s, also a second cytotoxic ILC1 population was identified, characterized as $CD127^{low}CD103^+$ ILC1s [326, 335, 355], and some ILC1s were found to lack the IL12Rb2 transcript [356]. In line with observations in human skin, intestines and nasal polyps [331, 357], lung ILC2s were defined by the expression of CRTH2. Lung

ILC3s were characterized as CD117 (c-kit) positive and a distinction was made based on the presence of the natural cytotoxicity receptor (NCR), i.e. NKp44, accordingly with studies in the gut and skin [337, 338]. Of note, NCR^- ILC3s are a heterogeneous population, including both NCR^- helper ILC3s as lymphoid tissue induces (LTi) cells [207]. For the future, it would therefore be interesting to expand our surface markers in order to distinguish between those two cell populations . Recently, neuropilin 1 (Nrp1) is suggested as an evolutionary conserved marker for human LTi cells [280].

Secondly, we confirmed that our staining based on specific surface markers adequately discriminated between the different ILC subsets, as a clear expression of specific developmental transcription factors could be shown. Of note, we observed that T-bet expression was not limited to the ILC1 population but was also present in ILC3s. In intestinal tissue, it was already shown that in response to IL-12 ILC3s can upregulate T-bet expression [335]. Further studies should however be conducted to investigate whether the observed T-bet expression in ILC3s is due to ILC plasticity.

Third, the expression of multiple cytokines, i.e. IFN-γ, IL-5, IL-17A, IL-22 and GM-CSF, was observed in the ILC population, indicative for the presence of all ILC subsets in human lung tissue. Especially the production of IFN- γ and GM-CSF seemed to be increased in human lung tissue. It would be interesting to explore these findings further as ILC3-driven GM-CSF production was indicated in intestinal homeostasis [220]. Moreover, it would be worthwhile to explore whether a shift would occur of one of these cytokine-expressing ILCs in a diseased state, such as COPD.

We have demonstrated that under homeostatic conditions all three ILC populations are present in human lung with ILC3s, especially NCR^- ILC3s, and ILC2s being the most prevalent. In line, predominantly ILC2s [358], that can be hold responsible for tissue homeostasis by expressing amphiregulin [357], were observed in normal skin tissue. Moreover, in the gut, IL-22-producing NCR^+ ILC3s which are responsible for maintaining gut homeostasis, accounted for about 70% of the total ILC population [335]. One can wonder if the observed ILCs at steady state will also contribute to lung homeostasis by producing high amount of GM-CSF or amphiregulin.

Interestingly, we demonstrated that in COPD GOLD I/II patients, the relative abundance of the ILC subsets tended to shift towards NCR^- ILCs. In line, a marked enrichment of NCR^- ILC3s was observed in lung tissue from patients with severe COPD (GOLD IV) compared to healthy controls and COPD GOLD I/II patients. Interestingly, recent advances detected ILC3s in inflammatory aggregates of COPD patients, indicative for a contribution of these ILCs in the formation of lymphoid aggregates and follicles [280]. In this context, functional studies to address the role of NCR^- ILC3s in COPD, and more specifically in lymphoid follicle formation would be an added value.

In our study, we were unable to find an accumulation of ILC1s in COPD patients, which was in contrast with COPD GOLD IV patients [278]. A possible explanation could be that we have studied less severe COPD patients and used a different gating strategy to characterize ILC1s compared to the study performed by Bal and colleagues.

11.3.3 Impact of glucocorticoids on ILCs

Since the 1940s corticosteroids are widely used in the clinic for their proficient anti-inflammatory effects. To data, inhaled corticosteroids also remain the most common and effective treatment for asthma [3, 359, 360]. Their clinical success is largely attributed to their trans-repressive effects in which they negatively interfere with the activation of DNA-bound transcription factors, leading to a reduced expression of multiple pro-inflammatory genes [361, 362]. Besides the well-known interplay between the glucocorticoid receptor and the pro-inflammatory transcription factors NF-κB and AP-1 [363], it was demonstrated that glucocorticoids also have potent inhibitory effects on T-bet and GATA-3 [364, 365], both involved in the development of T cells and ILCs [198].

In the treatment of allergic asthma, an important effect of glucocorticoids is the suppression of T_H2 cells and their typical type 2 cytokines, i.e. IL-4, IL-5 and IL-13 [359]. This suppressive effect of glucocorticoids can be mediated via the negative regulation of GATA-3, which is required for the transcription of T_H2 cytokine genes [366, 367]. Since besides T_H2 cells, also ILC2s depend on GATA-3, one can speculate that corticosteroids affect ILC2 function in the pathogenesis of allergic asthma. However, some *in vitro* studies indicated that ILC2s are more resistant to corticosteroids than T_H2 cells [231, 368], suggesting that the inhibitory effects of corticosteroids are not likely mediated by the modulation of ILCs.

Of note, in severe asthmatics, asthmatics who smoke or patients with COPD, decreased glucocorticoid responsiveness or even glucocorticoid-resistance is found [359, 360]. This reduction in corticoid responsiveness has often been ascribed to reduced expression of the glucocorticoid receptor or an altered affinity to its ligand/DNA-binding site [360]. Intriguingly, as the hypotheses was raised that especially ILCs would be of interest upon exposure to air pollutants [270], one could speculate that glucocorticoid insensitivity in these patients is mediated by the unresponsiveness of ILCs towards corticosteroids. In support of this, ILC2 numbers were still increased in the airways of severe asthmatics, despite high doses of oral corticosteroids [230]. Nevertheless, further studies should be conducted to address the controversy regarding the responsiveness of ILCs towards corticosteroids. An interesting train of thoughts could be that glucocorticoids can modulate ILC activity or influence ILC plasticity, as each

ILC subset has the capacity to change its phenotype and function dependent on the nature of signals they encounter in the tissue.

11.4 Potential implications to the patient

Asthma is a complex and heterogeneous disease with different clinical presentations and underlying disease processes. As patients respond different to therapy, asthma phenotyping has become utterly crucial. Moreover, a thorough understanding of the different asthma phenotypes will further improve the responses to targeted therapies [6]. Intriguingly, also in this dissertation, the importance of asthma phenotyping came forward and could have important implications to the patient in terms of therapeutic strategies.

First of all, targeting the chemerin/chemR23 axis could be of therapeutic interest for asthmatics considering its involvement in inflammatory cell recruitment. Given the anti-inflammatory actions of chemerin in the murine model of pollutant-aggravated allergic airway inflammation, chemerin agonists can be proposed as a novel therapeutic in the treatment of asthma. However, such chemerin agonists would be detrimental during a DEPs-induced lung inflammation in which chemerin exerts pro-inflammatory actions. Here, DEPs-induced inflammatory cell recruitment would rather be inhibited by blocking the chemerin/ChemR23 axis [369]. Based on these data, one can speculate that, in contrast to a non-allergic phenotype, allergic asthmatics could benefit from a chemerin agonist given their anti-inflammatory properties during allergen exposure. Nevertheless, before chemerin can even be considered as a new therapeutic, further research on the mechanisms underlying the opposing roles of chemerin should be conducted. Furthermore, also during the developmental stage of a chemerin agonist, caution is advised as different chemerin variants can exert entire opposite functions.

Secondly, both genetic and functional studies have identified IL-33 and its receptor as promising targets in the treatment of asthma [125, 153]. At the moment, IL-33 (AMG-282) and ST2 (CNTO-7160) blocking antibodies are in early clinical development [370]. In our study, prophylactically inhibiting the IL-33 signaling pathway decreased the pollutant-aggravated allergic airway inflammation. However, when the allergic airway inflammation was already established, blocking IL-33 signaling had no longer a therapeutic effect. In contrast to most allergic studies, our study creates doubt concerning IL-33 as a potential therapeutic target. More specifically, our data indicate that pollutant exposure can influence the treatment response of allergic asthmatics towards IL-33. We therefore suggest that knowledge concerning pollutant exposure should be included when phenotyping the patient. Of note, as we cannot exclude that the used dose,

application route or half-life of r-sST2 could have underestimated the potential of IL-33 as a new therapeutic, further studies should be conducted. At least recently, a press release stated that, in a phase 2 clinical trial, promising results were obtained with AMG-282 in atopic dermatitis patients [371].

Thirdly, in the past several years, a lot of researchers focussed on ILCs as possible therapeutic targets in asthma. However, in our model of DEPs-enhanced allergic airway inflammation, we demonstrated a crucial role for the adaptive system, rather than ILC2s [300]. In line, the hypothesis was raised that ILC2s would be of more importance in non-allergic asthmatics that were exposed to pollutants, microbes or glycolipids [270]. In support of this, increased corticosteroid-resistant ILC2s have been identified in the airways of severe asthmatics [230]. It should however be mentioned that also in allergic asthmatics, increased ILC2s can be demonstrated [233]. Of note, one can hypothesize that the contribution of ILCs in allergic asthma can be related to the properties of the allergen, as in papain-induced airway inflammation a prominent role for ILC2s was established [170]. Based on these findings, phenotyping the patients and identifying the underlying atopic responses are crucial for personalized treatment.

11.5 General Conclusion

In this dissertation, we have applied a model of combined exposure to air pollutants and allergens to investigate the involvement of epithelial-derived mediators, ILC2s and T_H2 cells during pollutant-enhanced allergic airway responses. We conclude that epithelial-derived chemerin and IL-33 are increased in response to combined DEPs+HDM. While neutralization of chemerin signaling aggravates the DEPs+HDM-induced airway inflammation, indicative for anti-inflammatory properties, deficiency in IL-33 signaling decreased the enhanced effects of DEPs on allergic airway inflammation in a prophylactic setting. Despite the fact that both ILC2s and T_H2 were activated upon combined DEPs+HDM exposure, only T_H2 cells appeared to be critical for the aggravating effects of DEPs on allergic airway inflammation, characterized by increased airway eosinophilia, mucus metaplasia, type 2 cytokine production, IgG1 production and AHR (Figure 20).

Furthermore, we demonstrated that all ILC subsets are present in human lung tissue and that their relative abundance can alter under pathological conditions, such as COPD. The observation of more NCR^- ILC3s in COPD patients could highlight an important role of this ILC subset during the pathogenesis of COPD.

CHAPTER 12: SUMMARY / SAMENVATTING

Summary

Worldwide, millions of people suffer from chronic respiratory diseases leading to a high socio-economic impact. The most common chronic airway diseases are asthma and chronic obstructive pulmonary disease (COPD) wherein both the airways and structural components of the lung are affected. Besides a genetic predisposition, several epidemiological and experimental studies have shown that exposure to air pollution can contribute to the induction and exacerbation of both asthma and COPD. Gaining insights into the molecular and cellular mechanisms underlying these pollutant-aggravated airway diseases is urgently needed for their prevention and treatment.

The airway epithelium is an important line of defence against a wide variety of environmental factors. In response to such foreign particles, the airway epithelial cells can release pro-inflammatory mediators that can attract and activate immune cells, such as dendritic cells (DCs) and innate lymphoid cells (ILCs), modulating inflammatory responses. In this dissertation, we used murine models to investigate how allergens (house dust mite, HDM) and air pollutants (diesel exhaust particles, DEPs) can modulate specific epithelial-derived mediators, leading to altered immune responses in the lung. Moreover, as a dysregulation of ILCs can be implicated in several inflammatory diseases, their contribution was investigated during the aggravating effects of DEPs on allergic airway inflammation and their abundance was investigated on *ex vivo* human lung samples.

In chapter 7, we have studied the role of chemerin, an important chemoattractant of DCs in DEPs-modulated immune responses. Preventing chemerin/ChemR23 signaling resulted in diminished inflammatory airway responses during acute DEPs exposure, whereas exaggerated allergic responses were demonstrated upon combined DEPs+HDM exposure. In chapter 8, the role of IL-33, an important innate pro-type 2 cytokine, implicated in the activation of both DCs and ILC2s, was investigated. By administering a recombinant decoy receptor (r-sST2) that neutralizes IL-33 activity, the involvement of the IL-33/ST2 signaling pathway in the onset of DEP-enhanced allergic airway inflammation was demonstrated.

In chapter 9, the role of ILC2s in DEPs-enhanced allergic airway inflammation was investigated. Whereas both ILC2s as T helper 2 (T_H2) cells can be implicated in the pathogenesis of asthma, little was known concerning their relative contribution during DEPs-enhanced allergic airway inflammation. First, we have demonstrated that both ILC2s and T_H2 cells were increased upon combined DEPs+HDM exposure. Moreover, the DEP-enhanced allergic airway responses were dependent on a GATA-3-mediated regulation of ILC2s and T_H2 cells. Finally, by using ILC2s or T_H2-

deficient mice, we demonstrated that predominantly T_H2 cells were critical to orchestrate the enhanced effects of DEPs on allergic airway inflammation and hyperresponsiveness.

Although research on ILCs has expanded rapidly the last decade, little was known concerning ILCs in the human respiratory system. In chapter 10, flow cytometric analyses on *ex vivo* human lung samples was performed to identify the different ILCs based on specific surface markers, transcription factors and cytokine production. We could demonstrate that all three ILC subsets are present in human lung tissue. Moreover, compared to control patients, we observed that the presence of the ILC subsets tended to change in patients with COPD, suggesting that they could be implicated in COPD pathogenesis.

Samenvatting

Wereldwijd lijden miljoenen mensen aan een chronische luchtwegaandoening wat een hoog sociaal economische impact met zich meebrengt. De meest voorkomende luchtwegaandoeningen zijn astma en chronisch obstructief longlijden (COPD) waarbij zowel de luchtwegen als structurele componenten van de long worden aangetast. Naast een genetische voorbeschiktheid, heeft epidemiologisch en experimenteel onderzoek reeds aangetoond dat voornamelijk blootstelling aan luchtverontreiniging bijdraagt aan de gestegen incidentie en ernst van zowel astma en COPD. Inzichten verwerven in de moleculaire en cellulaire mechanismen onderliggend aan de aggraverende werking van polluenten op luchtwegaandoeningen is dringend nodig voor een betere preventie en behandeling.

Het luchtwegepitheel is een belangrijke barrière voor diverse omgevingsfactoren. Na blootstelling aan vreemde partikels kunnen epitheelcellen diverse mediatoren vrijlaten die bijdragen aan de rekrutering en activatie van immuuncellen, zoals dendritische cellen (DCs) en aangeboren lymphoïde cellen (ILCs), de welke een modulerende effect op het immuunsysteem kunnen uitoefenen. In deze thesis hebben we muismodellen ontwikkeld om te onderzoeken op welke wijze allergenen (huisstofmijt, HDM) en polluenten (diesel uitlaatpartikels, DEPs) epitheliale mediatoren beïnvloeden en de immuun responsen in de long wijzigen. Aangezien een ontregeling van ILCs reeds werd aangetoond in diverse inflammatoire aandoeningen, werd bovendien de bijdrage van ILCs bestudeerd tijdens de aggraverende werking van polluenten op allergische luchtweginflammatie en hun voorkomen bestudeerd in humane longstalen.

In hoofdstuk 7, hebben we de rol van chemerine bestudeerd, een belangrijke chemoattractant van DCs in DEPs-geïnduceerde immuun responsen. Door de chemerine/ChemR23 signalering te verhinderen werd een daling van de inflammatoire luchtweg respons waargenomen na acute diesel blootstelling, in tegenstelling tot een gestegen allergische luchtweginflammatie na gecombineerd diesel en huisstofmijt blootstelling. In hoofdstuk 8, hebben we vervolgens de rol van IL-33 bestudeerd, een belangrijk cytokine betrokken bij de activatie van zowel DCs als ILC2s. Door toediening van een recombinante receptor die de IL-33 activiteit neutraliseert werd het belang van de IL-33/ST2 signalering aangetoond tijdens het aggraverend effect van DEPs op allergische luchtwegontsteking.

In hoofdstuk 9 werd de rol van ILC2s tijdens de aggraverende werking van DEPs op allergische immuun responsen onderzocht. Terwijl een prominente rol voor zowel ILC2s als T helper 2 (T_H2) cellen reeds werd aangetoond in de pathogenese van astma, was er weinig kennis voorhanden omtrent hun bijdrage tijdens het aggraverende effect van DEPs op allergische

luchtweginflammatie. Allereerst hebben we aangetoond dat zowel ILC2s als T_H2 cellen gestegen zijn na een gecombineerde DEPs+HDM blootstelling. Bovendien zijn de DEPs-geïnduceerde allergische luchtweg responsen afhankelijk van een GATA-3-gemedieërde regulatie van ILC2s en T_H2 cellen. Finaal, door gebruik te maken van proefdieren die deficiënt zijn in ILC2s of T_H2 cellen, hebben we aangetoond dat voornamelijk T_H2 cellen een cruciale rol vervullen tijdens de aggraverende werking van DEPs op allergische luchtwegontsteking en hyperresponsiviteit.

Hoewel de laatste jaren enorm veel onderzoek werd uitgevoerd naar ILCs, was er weinig kennis omtrent ILCs in het respiratoir stelsel van de mens. In hoofdstuk 10, hebben we flow cytometrische analyse uitgevoerd op humane long stalen met als doel de verschillende ILC subsets te identificeren aan de hand van specifieke oppervlakte merkers, transcriptiefactoren en cytokine productie. We konden aantonen dat alle ILC subsets aanwezig zijn in humaan long weefsel. Bovendien, in vergelijking met controle patiënten, konden we een wijziging in de frequentie van ILC waarnemen bij patiënten met COPD, indicatief dat deze cellen betrokken kunnen zijn in de pathogenese van COPD.

PART III: ADDENDUM

Review

References

Curriculum Vitae

Dankwoord

REVIEW: INSIGHTS IN PARTICULATE MATTER-INDUCED ALLERGIC AIRWAY INFLAMMATION: FOCUS ON THE EPITHELIUM

Exposure to air pollution is associated with many health problems, ranging from respiratory diseases to cardiovascular illness and all-cause mortality. In the lung, exposure to air pollution can lead to acute inflammation and modulate the onset and exacerbation of asthma. The mechanisms remain however largely unknown. We hypothesize that PM-induced cytokines are important mediators in the acute and aggravating effects of PM on airway inflammation. Targeting type-2 innate cytokines could therefore be a new approach in the treatment of asthma.

De Grove KC, Provoost S, Brusselle GG, Joos GF, Maes T. Insights in particulate matter-induced allergic airway inflammation: focus on the epithelium. Clinical and experimental allergy. 2018. IF: 5.264, ranking allergy: 4/26.

ABSTRACT

Outdoor air pollution is a major environmental health problem throughout the world. In particular, exposure to particulate matter (PM) has been associated with the development and exacerbation of several respiratory diseases, including asthma. Although the adverse health effects of PM have been demonstrated for many years, the underlying mechanisms have not been fully identified. In this review, we focus on the role of the lung epithelium and specifically highlight multiple cytokines in PM-induced respiratory responses. We describe the available literature on the topic including *in vitro* studies, findings in humans (i.e. observations in human cohorts, human controlled exposure and *ex vivo* studies), and *in vivo* animal studies. In brief, it has been shown that exposure to PM modulates the airway epithelium and promotes the production of several cytokines, including IL-1, IL-6, IL-8, IL-25, IL-33, TNF-α, TSLP and GM-CSF. Further, we propose that PM-induced type-2 promoting cytokines are important mediators in the acute and aggravating effects of PM on airway inflammation. Targeting these cytokines could therefore be a new approach in the treatment of asthma.

INTRODUCTION

Asthma is one of the most common chronic diseases worldwide, resulting in a serious global burden affecting as many as 334 million people [2, 4]. It is a chronic disease of the conducting airways characterized by a reversible airway obstruction, chronic airway inflammation, goblet cell metaplasia, airway hyperresponsiveness (AHR) and remodelling. Asthmatics suffer from symptomatic episodes of wheezing, coughing, breathlessness and chest tightness that vary over time and intensity [4].

Asthma is a complex and heterogeneous disease with different underlying disease processes. Multiple phenotypes can be distinguished based on the patient's clinical characteristics and inflammatory profile. The best characterized phenotype is (early-onset) allergic asthma, defined by the presence of allergen-specific immunoglobulin E (IgE) in serum and/or a positive skin prick test to common allergens, such as house dust mite (HDM), in association with type 2 mediated immune responses. Other phenotypes are i.e. late-onset eosinophilic asthma and smoking-associated neutrophilic asthma [6, 7]. Despite the heterogeneous nature of asthma, an almost identical pathological phenotype can be observed, characterized by an impaired epithelial barrier with areas of epithelial damage, excessive mucus production and thickening of the airway smooth muscles. Moreover, an excessive airway inflammation can be observed, which is a multicellular process involving mainly dendritic cells (DC), eosinophils, neutrophils, $CD4^+$ T lymphocytes and mast cells [5, 372].

There are several factors that can influence the development and phenotype of asthma **(Figure 1)** [3]. Twin studies have shown that there is a considerable genetic component in asthma, indicating that individuals can be genetically predisposed to be protected or more susceptible from developing asthma [14]. Moreover, genome wide association studies (GWAS) have identified several genetic polymorphisms related with the pathogenesis of asthma, such as TSLP and IL-33, implicating an important role for airway epithelial damage in the induction of airway inflammation [16, 17]. Despite the power of GWAS to identify disease-associated loci, a significant proportion of asthmatics do not carry the known asthma gene polymorphisms [18]. Furthermore, over the last century there has been a strong increase in asthma which cannot be explained by genetics but rather by gene-by-environment interactions [2]. Epigenetic changes for instance, including DNA methylation, histone modifications and non-coding RNAs, are considered important additional mechanisms in the development of asthma and are highly influenced by environmental exposures [20, 373, 374]. Moreover, it has been clearly demonstrated that exposure to a wide variety of environmental factors, such as allergens, respiratory infections and pollutants, are also important determinants in the onset and aggravation of asthma [2].

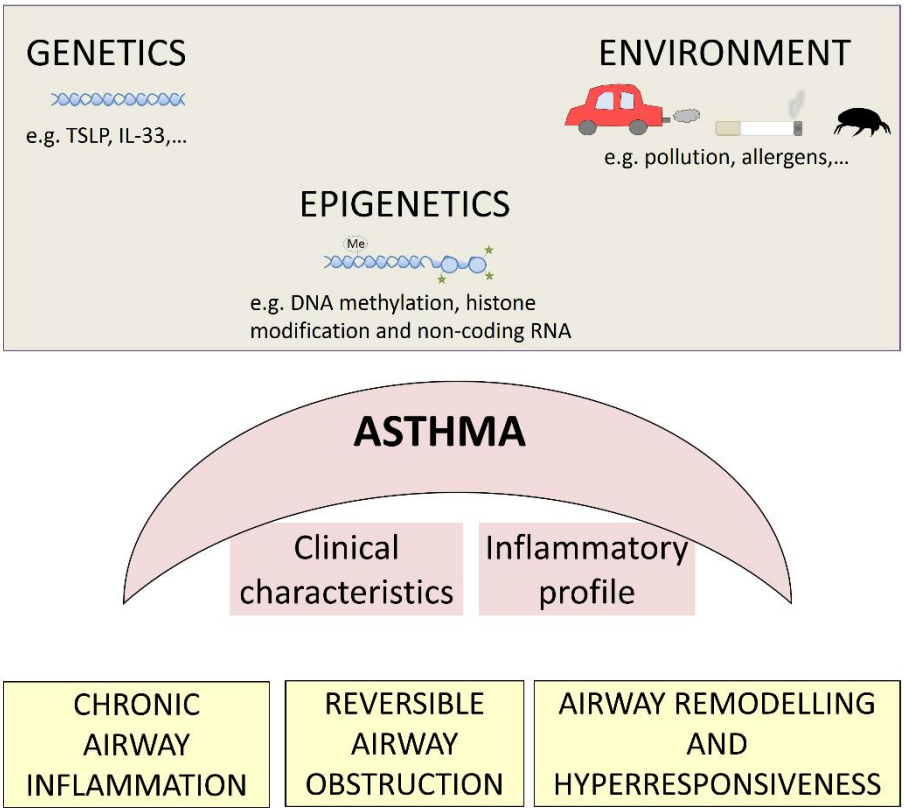


Figure 1: Risk factors contributing to the hallmarks of asthma. The development of asthma is strongly regulated by heritable components (genetics and epigenetic mechanisms) and exposure to environmental factors. Asthma is a heterogeneous disease wherein multiple phenotypes can be distinguished based on clinical characteristics and the inflammatory profile. The disease is being characterized by a chronic airway inflammation, reversible airway obstruction and airway wall remodelling and hyperresponsiveness.

AIR POLLUTION

Exposure to outdoor ambient air pollution represents a high environmental risk to human health, leading to approximately 3 million deaths each year [55]. Several epidemiological studies have demonstrated a positive correlation between air pollution and adverse health effects, ranging from respiratory diseases, such as asthma and chronic obstructive pulmonary disease (COPD), to cardiovascular illness and all-cause mortality, even at pollutant concentrations below the current national standards [60, 67-70]. This ambient pollution is a complex mixture of chemicals such as ozone, nitrogen dioxide (NO_2), sulphur dioxide (SO_2), biological substances and particulate matter (PM), derived from natural processes as well as from human activity, such as industry, cigarette smoke and traffic. Although all components of air pollution are harmful for human health, severe effects have been attributed to ambient PM since they can carry a broad range of toxic substances into the respiratory tract [56]. Ambient PM consists of solid and liquid particles that differ in their

chemical composition and are categorized based on their aerodynamic diameter (i.e. PM10, PM2.5 and PM0.1). When inhaled, PM_{10} (particles smaller than 10μM) deposits mainly in the nose and large conducting airways, whereas the deposition of $PM_{2.5}$ (particles < 2.5μM) is located throughout the respiratory tract, particularly in the small airways and alveoli. The ultrafine PM (UFPM) fraction $PM_{0.1}$ (particles < 0.1μM) will further deposit in the pulmonary tissue and some will even translocate from the alveoli to the pulmonary circulation [60]. A major component of traffic-related ambient PM are diesel exhaust particles (DEPs). These particles consist of a carbon core which can adsorb several organic compounds, such as polycyclic aromatic hydrocarbons (PAHs) and quinones. Additional, traces of sulphates, nitrates and metals can be observed. The majority of DEPs belong to the highly respirable fine or ultrafine PM fraction [64, 65].

Importantly, several key characteristics of ambient PM such as particle size, surface area and chemical composition will determine the specific mechanisms of PM-induced toxicological effects and their ability to induce lung injury and inflammation [57-59]. Emerging evidence has shown that, among different particles, especially UFPM has a great toxic potential due to the unique physical and chemical properties [59, 375, 376]. When the particle size decreases, the relative surface area increases, allowing a greater proportion of compounds to be displayed at the surface [57]. UFPM contains significantly more metals and a large number of organic carbon compounds compared to larger particles [63], which have been demonstrated to have pro-oxidant and pro-inflammatory effects in the respiratory system [61-63]. In addition, the high potency of UFPM to induce airway injury and inflammation is also due to a higher number of particles per mass unit, higher deposition efficiency and long-term retention/delayed clearance in the pulmonary region [377, 378]. Furthermore, several studies support the concept that UFPM is less likely to be phagocytized by alveolar macrophages and reside only a short period on the luminal side of the airway epithelium, penetrating deep into the tissue and cellular targets, lodge into the mitochondria, where cell injury, reactive oxygen species (ROS) generation and cell death can occur [379, 380]. Finally, the small size of UFPM facilitates its translocation from the respiratory epithelium towards the systemic circulation, causing extra-pulmonary health effects [378].

AIR POLLUTION AND THE AIRWAY EPITHELIUM

The airways are continuously exposed to particulate matter from the environment. Different mechanical filters located in the nasopharyngeal (i.e. nose hairs and the turbulence in the nasal turbinates), tracheobronchial and alveolar region protect the airways against inhaled foreign particles. Especially the mucociliary escalator is identified as a crucial self-clearing process of the airways to remove inhaled pathogens and particulates. More specifically, a large proportion of

foreign particles are being trapped in the mucus layer on top of the airway epithelium and transported out of the airways by ciliary beating and coughing [381, 382]. Importantly, it was demonstrated that PM-exposed human bronchial epithelial cells (HBEC) progressively attenuate the ciliary beat frequency [105], suggestive for a delayed clearance of these particles out of the lung. The particles that escape those defence mechanisms will then enter the lower airways and alveolar region where they can be endocytosed and removed by alveolar macrophages [381, 382]. Upon ambient PM exposure, these macrophages are activated and secrete an array of pro-inflammatory mediators, leading to apoptosis and induction of local and systemic inflammatory responses [383]. Interestingly, it was demonstrated that when exposed to ambient PM alveolar macrophages and airway epithelial cells can interact with each other to amplify the production of a variety of chemokines and cytokines, leading to inflammatory airway responses [384].

Initially, the airway epithelium was considered as a passive barrier which can exclude harmful agents from the underlying tissue by a dense network of tight and adherens junctions. In response to ambient PM exposure, this epithelial integrity can however be affected, as demonstrated by a downregulation or disruption of the adhesion molecule E-cadherin or tight junction proteins, occludin and zonula occludens-1 [107-109]. Nowadays, it is widely accepted that the epithelial layer also has an active role in the respiratory defence mechanisms, regulating both innate and adaptive immune responses. First of all, several studies have shown that besides the specialized phagocytotic cells, such as the alveolar macrophages, ambient PM can also be endocytosed by airway epithelial cells [385-387]. Although limited data is available concerning the exact underlying pathways, it is presumed that different uptake mechanisms can occur depending on the particle's characteristics [388, 389]. It was for instance demonstrated that the uptake of DEPs by lung epithelial cells was inhibited by cytochalasin D (CytoD), an inhibitor of actin-dependent uptake mechanisms, such as phagocytosis and micropinocytosis [390]. In contrast, uptake of UFPM was reported to be actin-independent [391]. Secondly, on the epithelial surface, different pattern recognition receptors (PRR) are expressed, which can be activated by pathogen-associated molecular patterns (PAMPs) in combination with the release of damage-associated molecular patterns (DAMPs) [102, 117, 392] **(Figure 2)**. Indeed, it was already demonstrated that ambient PM can trigger toll-like receptor (TLR)2 and TLR4. Both receptors signal through MyD88, which will lead to the translocation of NF-κB to the nucleus. Interestingly, depending on the particle's composition, distinct TLR may be activated. Until now, it is however unclear which components are responsible for TLR activation [115, 116, 393, 394]. Moreover, in addition to a TLR ligand, ambient PM can also influence TLR expression and their ability to respond to other ligands, leading to altered inflammatory responses to subsequent stimuli [393]. Besides TLR, also cytosolic NOD-like receptors (NLR), i.e. NLRP3 (NOD-like receptor, pyrin domain containing 3), can be activated through environmental air pollutants and endogenous danger signals [393].

However, there is still some controversy regarding the role of the NLPR3 inflammasome upon pollutant exposure [91, 395]. Alternatively, ambient PM can also enhance the expression and activation of different membrane-associated receptors, such as the epidermal growth factor receptor (EGFR) [396-401]. Dimerization of the EGFR will stimulate tyrosine kinase activity that can elicit PI3K/AKT signaling and mitogen-activated protein kinases (MAPK) (i.e. ERK, JNK and p38), both involved in the activation of the transcription factor NF-κB [61, 399, 402-405]. Importantly, after internalization or PRR activation, exposure to ambient PM/DEPs will activate oxidative stress responses in the airway epithelial cells. When lung epithelial cells are exposed to ambient PM/DEPs higher levels of intracellular ROS formation, activation of oxidative stress-responsive genes, such as HMOX1, and oxidative damage have been demonstrated [406-410]. Ambient PM/DEPs exposure was further associated with the overexpression of several genes and increased phosphorylated proteins related to the MAPK or PI3K/AKT pathway [61, 402-404, 406, 410-412]. Several studies also demonstrated that PM activates NF- κB signaling in airway epithelial cells to induce inflammation [410, 413-415].

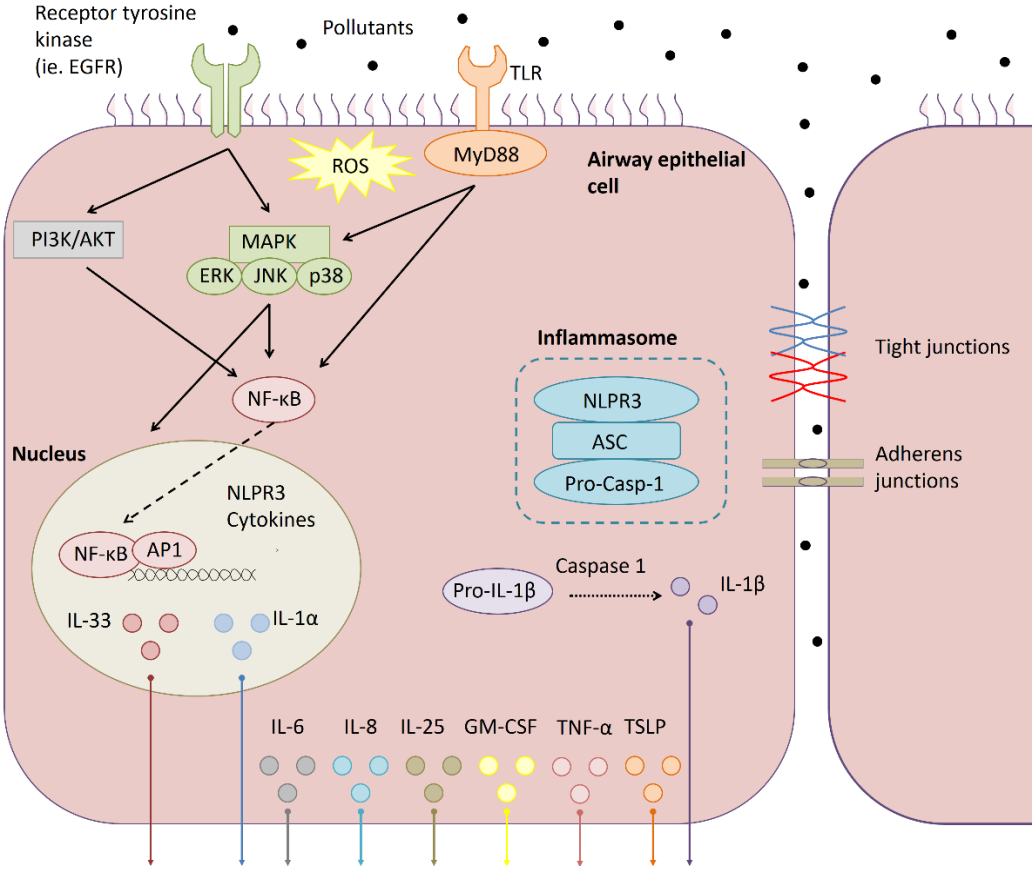


Figure 2: Mechanisms of PM-induced epithelial cytokine release. Exposure to PM induces production of ROS, epithelial disruption and activation of signalling pathways which are involved in the activation of NF-κB. Nuclear localization of NF-κB will then in turn lead to the transcription of NLRP3, involved in the NLPR3 inflammasome, and production of different cytokines. Both IL-33 and IL-1α are constitutively stored in the nucleus. Pro-IL-1β needs an additional cleavage to become functionally active. Conflicting results are obtained concerning the involvement of the NLPR3 inflammasome in the activation of pro-IL-1β. Symbols: → activation; - - → migration; ⌐→ transcription; ●→ secretion; ····→ cleavage

AIR POLLUTION AND ASTHMA

As stated above, DEPs can cause several adverse effects on their own, inducing acute inflammatory responses in the nose, throat and lungs [23]. Both human and animal studies have shown that ambient PM contributes to pulmonary inflammation through the induction of oxidative stress, influencing cell signaling pathways and production of inflammatory mediators, leading to inflammatory cell recruitment [90, 92, 94, 416]. Ambient PM inhalation also affects the autonomic nervous system (i.e. modulates heart rate variability [417]) and induces bronchoconstriction and cough by sensory nerve activation [418, 419]. In addition, ambient PM inhalation initiates airway inflammation by activation of irritant receptors and acid-sensitive pathways located on sensory C nerve fibers lining the nasal and pulmonary airways. This results in the release of neuropeptides, like substance P (SP), leading to the initiation and maintenance of PM-mediated airway inflammation [420]. In both human asthmatics and rodent models, it has been suggested that exposure to DEPs augments neuropeptide-release through sensory nerve activation, leading to increased adverse effects of ambient PM on respiratory diseases [421, 422].

Various epidemiological studies provided evidence that exposure to traffic-related air pollution can cause as well as exacerbate both childhood and adult-onset asthma [60, 78-81]. Living near major roads was for instance associated with significantly more respiratory symptoms and adverse effects on lung function in children and adults [70, 82-85]. Moreover, when asthmatic individuals were exposed to high levels of particulate pollution, they were more likely to experience worsened symptoms and impaired lung function, leading to uncontrolled asthma and hospitalizations [79, 81, 87]. Controlled human exposure studies further confirmed that allergen-specific responses, such as airway eosinophilia, type 2 cytokine expression and allergen-specific IgE, can be augmented when the individuals were exposed to DEPs and an (neo)-allergen [99-101].

Interestingly, the susceptibility towards the adverse effects of PM inhalation is dependent on genetic factors [423]. Polymorphisms in genes encoding for enzymes that regulate oxidative stress, *GSTM1*, *GSTP1* and *NQO1*, are identified as risk factors for allergic diseases in response to DEPs [424]. High DEPs exposure during infancy for instance could be related to an increased risk of wheezing among carriers with GSTP1 polymorphism (*GSTP1* Val105) [425]. Furthermore, the aggravating effect of DEPs on allergic airway inflammation, i.e. IgE and histamine production, was enhanced when individuals had single nucleotide polymorphisms (SNPs) in the *GSTM1* and *GSTP1* gene [426]. Moreover, a *GSTM1* variant status led to decreased FEV1 levels following DEPs and allergen co-exposure [427]. Finally, also genetic variants in the *TLR2* and *TLR4* genes influence the susceptibility to adverse effects of traffic-related ambient PM on childhood asthma [428].

By modulating various airway epithelial responses, i.e. endocytotic uptake, ROS generation and PRR activation, it is suggested that pollutants can initiate or contribute to the pathological features of asthma [23]. Below, we will elaborate on the modulating effects of ambient PM, and in more detail of DEPs, on cytokine expression. In a first part, we will summarize the influence of ambient PM/DEPs on multiple cytokines in human airway epithelial cells *in vitro*. Secondly, we will highlight some type 2 mediated cytokines that have been shown to be involved in allergic asthma, including also human studies and *in vivo* animal studies. The strengths and limitations [23, 429, 430] of the different research approaches are summarized in **Table 1**. PubMed and Web of Science were searched using "particulate matter" or "diesel exhaust particles" in combination with "IL-1 OR IL-6 OR IL-8 OR IL-25 OR IL-33 OR GM-CSF OR TNF-α OR TSLP". A filter for research in the lung was applied. Additional relevant references were obtained from the reference list of retrieved manuscripts. Publications in English were considered until the 18th of January 2018.

Table 1: Advantages and limitations of basic and translational research to study the impact of PM on airway inflammation

	Advantages	**Limitations**
Epidemiological studies	Large cohorts 'Real world' exposure Mortality and morbidity endpoints Study acute and chronic effects Study susceptible populations	Limited material (mostly blood) Estimation PM exposure Confounding factors/exposures Difficult to demonstrate causality
Controlled human exposure studies	Well defined PM exposure Control confounding exposures Various endpoints possible Intervention studies	Ethical issues Small subject numbers Exclusion of susceptible populations
***In vitro* studies**	Investigate specific cells of interest Possibility of mechanistic and/or intervention studies	Different from the *in vivo* situation: artificial milieu with limited cell to cell interactions Difficult to mimic *in vivo* exposure
Rodent *in vivo* studies	Complete living organisms Mimic several asthmatic parameters	Ethical issues

	Possibility of mechanistic and/or intervention studies Fully sequenced and highly homologue genome with humans Relatively easily housed, bred and handled	Differences in respiratory anatomy, physiology and immune system Difficult to mimic real life exposure

PARTICULATE MATTER AND EPITHELIAL CYTOKINE EXPRESSION: HUMAN *IN VITRO* STUDIES (Supplemental Data – Table S1)

To evaluate the pro-inflammatory responses induced by ambient PM and DEPs *in vitro*, the epithelial gene expression and protein release can be measured. When HBEC, derived from immortalized cell lines or normal donors, are exposed to ambient PM or DEPs, a strong upregulation of different epithelial cytokines can be observed. The levels of IL-6 [406, 408, 410, 411, 431-438], IL-8 [115, 385, 404, 407, 410-412, 414, 432, 434, 436-443] and TNF-α [432, 444] for instance were significantly increased in cells that were exposed to PM. Of note, also MIP-3α/CCL20 [440, 445], an important chemokine involved in DC recruitment, was increased in HBEC upon ambient PM exposure.

Over the years, a triad of epithelial-derived cytokines, i.e. interleukin (IL)-25, IL-33 and thymic stromal lymphopoietin (TSLP), have been identified as crucial regulators of various immune cells that contribute to the pathophysiology of asthma [306, 446, 447]. In support of this, genome wide association studies (GWAS) identified that polymorphisms in genes encoding for TSLP, IL-33 and its receptor IL1RL1 are associated with asthma risk [16, 17]. Several studies demonstrated increased TSLP and IL-33 expression in the airways of asthmatics, which positively correlated with disease severity [124-126]. Moreover, in a subset of asthmatics, an increased epithelial expression of IL-25, which correlated with IL-25 plasma levels, could be demonstrated. Interestingly, no increase of IL-33 or TSLP was found in these patients [223], indicating the heterogeneity and complexity of the disease. In addition, IL-1 and granulocyte colony stimulating factor (GM-CSF) have also been identified as important type-2 promoting cytokines [117] and were both increased in the bronchial epithelium of asthmatics [448, 449]. Interestingly, these typical type 2 promoting cytokines, including, IL-1α [411, 440], IL-1β [395, 404, 410, 432, 434, 435, 437, 440, 450], IL-33 [123], TSLP [122] and GM-CSF [385, 403, 432, 439, 440, 451, 452], were also increased in PM-exposed normal HBEC. In contrast, IL-25 levels were undetectable [452] or decreased [185]. Importantly, the number of studies regarding the modulating effects of PM on

asthmatic HBEC are limited and further research is needed. Considering that asthmatic epithelium is more susceptible towards oxidants that can affect apoptosis and epithelial damage, compared to normal airway epithelium [104, 453], epithelial cells from asthmatics could be more sensitive towards the oxidative effects of PM. For the moment, this hypothesis is supported by the fact that elevated IL-33 production could be observed in PM-exposed asthmatic-derived epithelial cells whereas this was not the case for normal HBEC that were exposed to PM [185]. Of note, as the IL-33 function can be modified by oxidation [148], it could be that PM-generated ROS and oxidative stress could have important implications on IL-33 oxidation and subsequently its function. Furthermore, in asthmatic epithelium pro-inflammatory cytokines are constitutively more present and further increase upon low PM concentrations whereas in normal HBEC only the higher PM levels increased the release of IL-8 and GM-CSF [454]. Asthmatic ALI epithelial cell cultures also enhanced inflammatory responses towards PM, including increased IL-6, IL-8 and GM-CSF release, compared to non-asthmatic ALI cultures that were exposed to PM [455]. Further research where HBEC are exposed to PM in combination with allergens could be an interesting tool to investigate the aggravating effects of pollutants on allergen-induced epithelial responses.

Although it is clear that DEPs and PM can upregulate the expression of numerous pro-inflammatory epithelial cytokines, not all studies reported an altered cytokine expression [406, 408, 411, 452]. Differences in epithelial responses can be explained by variations in chemical composition, more specifically by the amount of adsorbed organic compounds, metals and presence of endotoxins on the particle's surface. By extracting ambient PM or DEPs for instance, it was demonstrated that especially the organic compounds were the main contributors to epithelial cytokines release, i.e. IL-1β, IL-6, IL-8 and GM-CSF [61, 402, 434, 456, 457]. Moreover, employment of metal chelators led to a significant decrease in IL-1α, IL-8 and GM-CSF expression, indicative for the contribution of metals in cytokine production [407, 440]. Removal of surface-bound endotoxins led to decreased cytokine responses, i.e. IL-1α and GM-CSF [440, 458], implicating endotoxins in the PM-induced epithelial cytokine release. In line, epithelial responses differed when sample collection was performed over different time periods or locations [403, 450, 459, 460]. Furthermore, epithelial cytokine expression can be influenced by the particle's size [115, 403, 441, 458, 461], as well as the experimental set-up, the selected PM dose and epithelial cell culture [433, 441, 461-463].

HOW DOES PARTICULATE MATTER INFLUENCE EPITHELIAL CYTOKINE EXPRESSION?

Mechanistic studies have already provided some insights on how pollutants modulate epithelial cytokine expression **(Figure 2)**. Endocytotic uptake mechanisms or activation of PPR for instance

are required for the induction of airway epithelial cytokine release upon PM exposure. Addition of CytoD, an inhibitor of actin-dependent uptake mechanisms, resulted in a significant reduction of PM-induced IL-8 and GM-CSF levels [402, 407], whereas a blocking antibody to TLR2 inhibited the PM-induced IL-8 expression [115]. Moreover, DEPs-induced IL-8 and GM-CSF release was blocked by neutralizing antibodies towards EGFR or autocrine ligands of the EGFR, indicating that activation of the EGFR is an important pathways in DEPs-induced cytokine release [396, 412, 464]. A crucial role for ROS formation during epithelial cytokine production was also identified, as different PM-induced cytokines, i.e. IL-1α, IL-1β, IL-6, IL-8 and GM-CSF, were significantly decreased after treatment with radical scavengers/antioxidants [61, 399, 402, 407, 410, 433, 440].

Exposure to pollutants can lead to the activation of several signaling pathways which will modulate cytokine gene expression. Upon DEPs exposure, the upregulation of epithelial cytokines could be attributed to de novo protein and mRNA synthesis [402, 439]. Moreover, signal transduction via the MAPK or PI3K pathway was found to be crucial for cytokine production, as treatment with specific tyrosine kinases, MAPK or PI3K inhibitors led to a diminished release of IL-1β, IL-6, IL-8 and GM-CSF in DEPs-exposed HBEC [61, 399, 402-404, 410]. Furthermore, by using specific NF-κB inhibitors or luciferase reporter gene assays, it was demonstrated that PM-induced NF-κB activation controls IL-1β, IL-6 and IL-8 production [410, 414, 433]. Importantly, the production of ROS has been associated with the redox-sensitive transcription factor NF-κB and the upstream signaling pathways. Pretreatment with antioxidants significantly inhibited DEP-induced ERK/AKT phosphorylation, NF-κB activation, and subsequent cytokine production, i.e. IL-1β, IL-6, IL-8 and GM-CSF [61, 404, 410, 414, 433]. Overall, one can state that the PM-induced inflammatory responses, such as cytokine production, are predominantly mediated by the NF-κB pathway which is activated by ROS and MAPK. Of note, dependent on the amount of organic compounds that are present on DEPs, a differential transcriptional regulation of IL-8 expression was proposed. Whereas exposure to DEPs with a lower organic content involved the NF-κB pathways to induce IL-8 promotor activity, high organic DEPs were associated with an AP-1-dependent mechanism [465].

However, identifying additional upstream signaling pathways that regulate cytokine production in response to pollutants is of great interest for new intervention strategies. Recently, a novel mechanism was proposed whereby the expression of TSLP was regulated by miRNA-375 via the aryl hydrocarbon receptor (AhR). More specifically, it was suggested that the suppressive effects of Ahr on NF-κB's transcriptional activity are downregulated by miRNA-375, leading to increased TSLP expression [466]. Moreover, increased IL-6 and IL-8 production upon PM exposure was suggested to depend on a sustained activation of NF-κB, which was regulated by a ROS/PI3K/AKT-

dependent downregulation of miRNA-331 expression in airway epithelial cells [415]. Notably, mechanistic insights concerning the altered expression of typical type-2 promoting cytokines, i.e. IL-25, IL-33 and TSLP, towards PM exposure are highly lacking.

PARTICULATE MATTER AND TYPE-2 PROMOTING CYTOKINES: EXPRESSION AND MECHANISMS IN HUMANS AND MURINE *IN VIVO* MODELS (Supplemental Data - Table S2 and S3)

Considering that in the pathogenesis of allergic asthma, typical type-2 promoting cytokines, i.e. IL-1, IL-25, IL-33, GM-CSF and TSLP, have been implicated, we will now further elucidate on their role using human studies and *in vivo* animal studies.

By collecting data from PM measuring stations, estimating the proximity to major roads or performing controlled human exposure and *ex vivo* studies, associations between pollution exposure and inflammatory markers are being explored in humans. When IL-1β or GM-CSF was measured locally, in exhaled breath, lavage fluid or bronchial tissue, no associations with PM could be observed [467-469].The effect of PM on local cytokine release is however an underexplored research field, with a limited number of studies available. In contrast, most human studies investigate the effect of air pollution using blood, in which the identified cytokines can have either an epithelial or hematopoietic origin. Notably, exposure to PM has generally been related to an increased expression of IL-1β in the blood of adults, children and during prenatal life [470-477]. One study also observed a correlation between a joint increase in TSLP+IL-33 cord blood levels and heavy street traffic during pregnancy [478] **(see table S2 for a detailed overview)**.

In vivo research in mice, i.e. using specific knockout strains and monoclonal antibodies, can provide additional insights into potential mechanisms how air pollution can modulate (allergic) airway inflammation and offer the opportunity to validate hypotheses that have been generated by human studies. First, during acute DEPs-induced inflammatory lung responses, an increased expression of IL-1β [91, 395, 400, 479-487] and GM-CSF [89, 452] in BALF and lung tissue were observed, whereas 1 study reported IL-25, IL-33 and TSLP as undetectable [452]. Moreover, the increased IL-1β and GM-CSF levels could be linked to PM or DEPs-induced inflammation and AHR [89, 91, 485]. Interestingly, using a mouse model of acute DEP-induced lung inflammation, IL-1β/IL-1RI signaling was identified as a critical regulatory pathway of the inflammatory responses towards DEPs [91]. Some murine *in vivo* studies however did not demonstrate an upregulation of IL-1β [483, 488] or GM-CSF [479, 489] after acute PM or DEPs exposure, possibly due to different exposure protocols, time of sampling, murine strains and pollutant composition. Secondly, in

murine models of DEPs-enhanced allergic airway inflammation, increased pulmonary levels of IL-1α [490] and GM-CSF [490, 491] in OVA models and higher IL-25 [300], IL-33 [300] and GM-CSF [492] levels in the HDM models were observed. In other studies however, similar or decreased GM-CSF levels were found in DEPs+OVA-exposed mice compared to sole OVA exposure [186, 493-497] or were undetectable during DEPs+HDM administration [498] **(see table S3 for a detailed overview)**. Notably, in these studies the exact cellular origin of these cytokines was however not determined. Moreover, to date, most studies only focused on cytokine expression, whereas mechanistic studies to unravel the contribution of these type-2 promoting cytokines in DEPs-induced development and aggravation of allergic asthma are scarce.

Besides the release of typical type-2 promoting cytokines, also chemokines, like CCL2/MCP-1, CCL20/MIP-3α and chemerin, are released upon DEPs exposure, contributing to recruitment of DC towards the pulmonary tissue [94, 95, 369]. DEPs are internalized by DC, that in response to the type-2 promoting cytokines will mature and migrate towards the MLN [94, 122, 123, 451], which was found to occur in a CCR7-dependent manner [94]. DEPs can thereby bind allergens onto their surface, acting as carriers to increase allergen deposition. Moreover, co-administration of allergens and pollutants can facilitate the penetration of allergens into the airway mucosa, resulting in a higher recruitment, uptake, maturation and migration of these DC [23, 499]. In the mediastinal lymph nodes, DEPs can affect the responses towards allergens by enhancing T_H2 cell polarization [94, 122, 123, 451]. In this manner, DEPs can increase the allergic responses, characterized by increased inflammatory cell influx, type 2 cytokine production, immunoglobulin production, mucus metaplasia and AHR [23, 300, 369, 500] **(Figure 3)**. In addition to the modulating effects of DEPs on the DC-T_H2 pathway, also innate lymphoid cells type 2 (ILC2) can become activated by the secretion of some specific type-2 promoting cytokines [198, 199]. Recently, it was however demonstrated that the aggravating effect of DEPs on allergic airway inflammation was predominantly dependent on the adaptive immune system, rather than ILC2 [300]. In addition to the effects of pollutants on asthma development, murine exacerbations models can be used to evaluate the aggravating effects of PM or DEPs on allergen-induced airway inflammation [23]. Interference with anti-IL-33 for instance suggested a role for IL-33 during an acute PM-induced exacerbation in a model of established chronic allergic asthma [186].

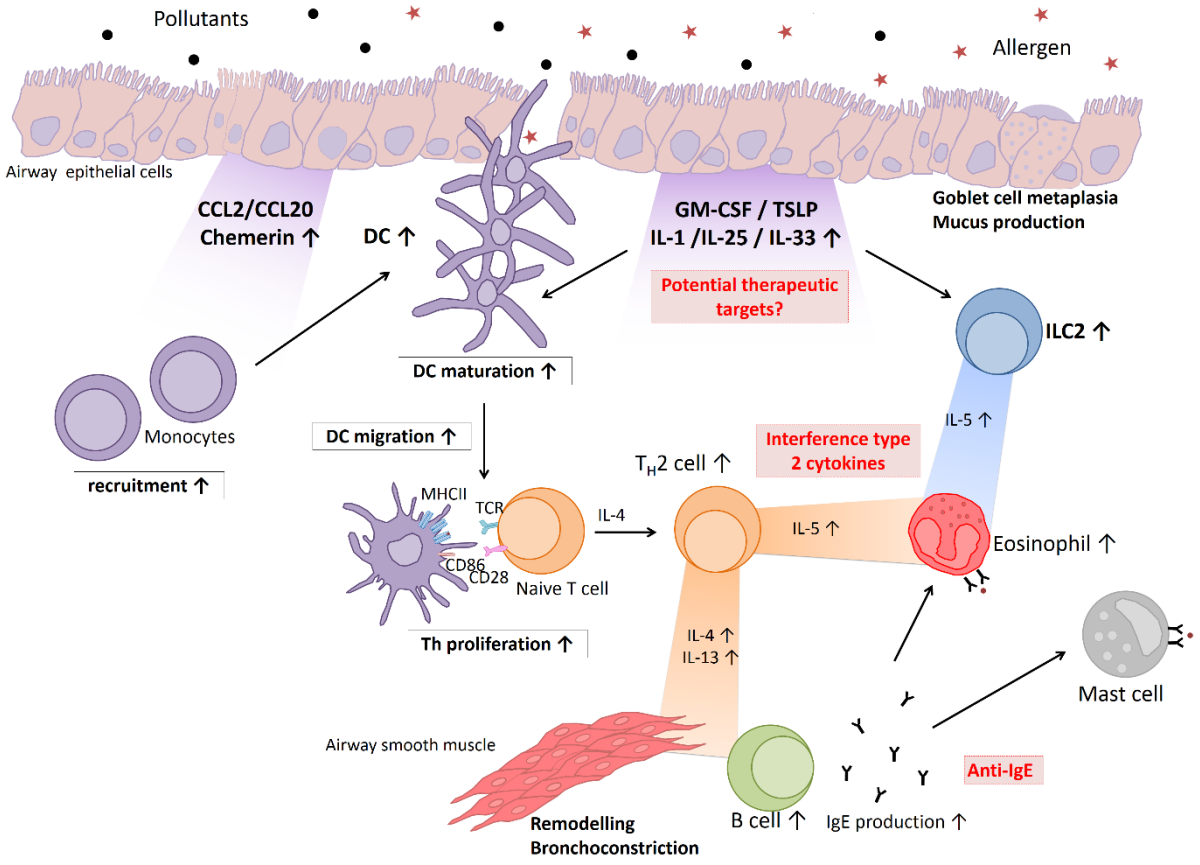


Figure 3: Diesel exhaust particles affect epithelial cytokines in the pathogenesis of allergic airway inflammation. Exposure to DEPs will activate the airway epithelium to secrete specific chemokines, leading to an increased recruitment of monocyte-derived DC to the pulmonary tissue. Specific epithelial-derived cytokines in combination with allergen and pollutant uptake will result in increased DC maturation, migration towards the MLN and induction of Th2 responses. The same epithelial-derived cytokines will also activate ILC2. These Th2 cells and ILC2 will then in turn secrete specific type 2 cytokines inducing airway eosinophilia, mucus metaplasia, airway wall remodelling and airway hyperresponsiveness. Cells and mediators that increase upon combined allergen and pollutant exposure are indicated with a black arrow↑. ● = pollutants, ★ = allergen, CCL = C-C motive chemokine ligand, DC = dendritic cell, IgE = Immunoglobulin E, IL = interleukin, ILC2 = innate lymphoid cell type 2, Th = T helper. Therapeutic targets are indicated in a red box.

Despite many asthmatic features, i.e. pulmonary inflammation, remodelling and AHR, that can be replicated in murine models, also important interspecies differences in immunology, respiratory physiology and anatomy should be taken into account [23, 501]. The expression of human IL-33 for instance is mainly localized in bronchial epithelial cells whereas in the mouse IL-33 is predominantly expressed by alveolar type II pneumocytes [139], which may be important when investigating the effects of air pollution in *in vivo* models. Moreover, differences in particle distribution due to a distinct pulmonary lobulation, bronchial branching and altered clearance and filtering processes can affect the pulmonary responses towards PM exposure [23]. Caution is therefore advised when extrapolating results obtained from murine models to the human

situation. Furthermore, some general differences between human and murine studies have been summarized in **table 1**.

POTENTIAL THERAPEUTIC TARGETS IN THE TREATMENT OF POLLUTANT-INDUCED AIRWAY INFLAMMATION

In addition to standard therapy, poor-controlled, severe asthmatics with a specific asthmatic phenotype are treated with a few approved biologics, i.e. anti-IgE (omalizumab) or anti-IL-5 (mepolizumab or reslizumab)) [502]. Moreover, also several biologics that target the type-2 promoting cytokines, i.e. anti-TSLP (Tezepelumab) [50], anti-GM-CSF [503] and anti-IL-33 [169], have entered (pre)clinical trials to evaluate their efficacy as add-on therapy in asthmatics. Intriguingly, as severe asthmatic phenotypes are associated with exposure to air pollutants, microbes and glycolipids [270], this raises the hypothesis that these biologicals would also be beneficial within the context of PM exposure. Furthermore, considering that exposure to air pollution can lead to the development of neutrophilic asthma [6] and reducing IL-1β levels diminished airway neutrophilia, targeting IL-1 signalling pathways could also be beneficial [504].

Besides modifying the expression of type-2 promoting cytokines, implications of PM on allergic inflammation and asthma exacerbations are for a significant part due to the production of ROS [505]. As pretreatment with antioxidants can affect intracellular signaling pathways and decrease cytokine production [61, 404, 410, 414, 433], enhancing the levels of the antioxidant superoxide dismutase (SOD) or influencing the glutathione (GSH) system for instance could be a another approach to lower oxidative stress and airway inflammation [506-509]. Especially since both asthma and PM exposure have already been associated with a significant reduction in SOD or polymorphisms in genes implicated in the GSH system [424, 426, 510-512]. Moreover, also dietary antioxidants [508, 513], such as selenium, were already associated with reduced oxidative stress and lung inflammation in a model of PM-induced lung injury [487]. Furthermore, selenium supplementation improved symptoms in asthmatics compared to placebo [514].

CONCLUSIONS AND FUTURE PERSPECTIVES

Although PM is without a doubt associated with an increased risk of asthma development and exacerbations, specific mechanisms are incompletely known. Epidemiological studies, as well as the use of both *in vitro* and *in vivo* models, have revealed a central role for the airway epithelium and type-2 promoting cytokines during pollutant exposure. The modulating effects of particulate matter on multiple cytokines can therefore be proposed as an important driver of allergen-induced airway responses. It is however crucial that the interaction of multiple environmental factors and the mechanisms involved are further elucidated. Additional research on the role of type-2 promoting cytokines during pollutant-enhanced airway inflammation may lead to the discovery of better therapeutics in the treatment of asthma.

SUPPLEMENTAL TABLES

TABLE S1: PARTICULATE MATTER AND EPITHELIAL CYTOKINE EXPRESSION: HUMAN *IN VITRO* STUDIES				
Material		**Exposure protocol**	**Observations epithelial cytokines**	**Reference**
IMMORTALZED CELL LINE	16HBE14o- cells	DEPs (SRM 1650) and CB (carbon black) at 10 µg/cm² for 6- 48h	Time-dependent DEPs-induced ↑ IL-8 and GM-CSF secretion. CB ineffective.	Boland et al., 1999
	BEAS-2B cell line	0.1-100 µg/mL DEPs for 24h	Time and dose dependent ↑ IL-8 mRNA expression upon DEPs exposure Involvement ROS and NF-κB	Takizawa et al., 1999
	16HBE14o- cells	2.5-20 µg/cm² DEPs (SRM 1650) for 6-48h	Time and dose dependent ↑ GM-CSF release in the presence of DEPs Importance organic compounds (lower effect of extracted DEPs and CB ineffective), ROS, tyrosine kinases and *de novo* protein synthesis	Boland et al., 2000
	16HBE14o- cells	10 µg/cm² DEPs, organic extracts of DEPs, stripped DEPs (SRM 1650) or carbon black for 24h	↑ GM-CSF secretion by DEPs and organic extracts of DEPs. Importance organic compounds (lower effect of stripped DEP and no effect of CB), involvement of ROS and MAPK pathways	Bonvallot et al., 2001
	BEAS-2B cell line	100 µg/mL PM at different locations for 4-16h	↑ IL-6 release by all PM samples	Veronesi et al., 2002
	16HBE14o- cells	DEPs (SRM 1650), RPM (SRM 1648), atmospheric $PM_{2.5}$ and CB at 10-30 µg/cm² for 24h	Dose dependent ↑ GM-CSF release after DEP/PM exposure. DEPs had a higher effect compared to PM. CB are ineffective.	Baulig et al., 2003
	16HBE14o- cells	10 µg/cm² multiple $PM_{2.5}$ samples/DEPs for 24h	↑ GM-CSF and TNF-α release for all PM/DEPs samples	Baulig et al., 2004

	16HBE14o- cells	$PM_{2.5}$ and DEPs (SRM1650) at 10 µg/cm² for 24h	Causal relationship between amphiregulin and GM-CSF production in response to DEPs and PM	Blanchet et al., 2004
	L132 cell line	$PM_{2.5}$ at 18-75 µg/mL for 24- 72h	Time and dose dependent $PM_{2.5}$-induced ↑ IL-1β, IL-6, IL-8, TNF-α release and IL-1β, IL-6, IL-8, TNF-α and GM-CSF mRNA expression	Dagher et al., 2005
	16HBE14o- cells	$PM_{2.5}$ at 10 µg/cm² for 24h	↑ GM-CSF protein levels after PM exposure EGFR ligands involved in GM-CSF release	Rumelhard et al., 2007
	16HBE14o- cells	Coarse, intermediate, fine and ultrafine PM for 24h at 1 or 10 µg/cm²	Dose dependent ↑ GM-CSF release following PM exposure. GM-CSF levels decreased with increasing aerosol size Role for endotoxins	Ramgolam et al., 2009
	16HBE14o- cells	DEPs (SRM1650), CB or $PM_{2.5}$ samples at 10 µg/cm² for 24h	↑ GM-CSF protein after treatment with DEPs and $PM_{2.5}$ from all sites. CB are ineffective ROS induction, EGFR and MAP kinases signalling pathways involved in the GM-CSF release.	Baulig et al., 2009
	BEAS-2B cell line	1.25-20 µg/cm² coarse and fine PM or 5-80 µg/cm² SRM1648 for 24h	Dose-dependent ↑ IL-8 release following coarse PM and SRM1648. No response towards fine PM	Ovrevik et al., 2009
	BEAS-2B cell line	Organic $PM_{2.5}$ at 1-100 µg/mL for 24h	Polar organic extracts induce dose-dependent ↑ IL-1β secretion	Fuentes-Mattei et al., 2010
	BEAS-2B cell line	10 µg/cm² DEPs with varying organic content for 4h	↑ IL-8 mRNA expression with DEPs exposure to low and high organic content	Tal et al., 2010
	BEAS-2B cell line	Different PM samples at 3-12 µg/cm² for 24-72h	Dose-dependent ↑ IL-1β, IL-6 and IL-8 mRNA levels for all different PM samples. No differences in TNF-α mRNA ↑ IL-6, IL-8 and TNF-α protein levels in the three PM samples. No difference in IL-1β protein levels	Dieme et al., 2012

	16HBE14o- cells	$PM_{2.5}$ at 0.1-10 µg/cm² for 2- 48h	Time-dependent $PM_{2.5}$-induced GM-CSF ↑ at both mRNA and protein level GM-CSF release dependent on autocrine effect of EGFR ligands	Ramgolam et al., 2012
	BEAS-2B cell line	12.5 or 25 µg/mL $PM_{2.5}$ or PM_{10} 6-24h	$PM_{2.5}$ -induced ↑ IL-1α and IL-6 release, ↑IL-8 only after 24h IL-1β was undetectable	Watterson et al., 2012
	BEAS-2B cell line	100 µg/mL DEPs for 4h	↑ IL-1β, IL-6 and IL-8 mRNA and protein levels when exposed to DEPs	Totlandsdal et al., 2012
	BEAS-2B cell line	100 µg/mL DEP for 2,4 or 6h	↑ IL-6 and IL-8 mRNA expression after DEP exposure	Bach et al., 2014
	HBEC (HBEC-6KT)	500 µg/mL PM_{10} (EHC-93) for 24h	PM_{10}-induced↑ IL-1β and GM-CSF production. IL-1β release proceeds GM-CSF production. No detection of TSLP, IL-25 and IL-33 NLRP3 inflammasome-dependent	Hirota et al., 2015
	16HBE cells	$PM_{2.5}$ at 25-150 µg/mL for 5 and 24h	Dose and time-dependent ↑ IL-6 production Decrease in IL-8 and TNF-α	Zhou et al., 2015
	BEAS-2B cell line	100 µg/mL $PM_{2.5}$ from six cities of China for 24h	↑ IL-1β mRNA levels by all $PM_{2.5}$ samples Association with concentration of metals (Ni) on PM	Yang et al., 2016
	BEAS-2B cell line	2.5-10 µg/cm² $PM_{2.5}$ and 10 µg/cm² DEPs for 24h	Dose dependent ↑ IL-8 release after PM/DEPs exposure Involvement ROS and endocytosis Importance metals	Yan et al., 2016
	BEAS-2B cell line	100 µg/mL PM_{10} from three different locations	↑ IL-8 release by all PM samples	Van Den Heuvel et al., 2016
	BEAS-2B cell line	2µg/cm² $PM_{2.5}$ for 72h	↑ IL-6 and IL-8 production following PM exposure	Leclercq et al., 2017

	BEAS-2B cell line	5µg/cm^2 diesel UFP for 20h	↑ IL-6 release after DEP No difference in IL-8 and TNF-α Importance ROS and MAPK related genes	Bengalli et al., 2017
HUMAN BRONCHIAL EPITHELIAL CELLS (HBEC) NORMAL SUBJECTS	HBEC from normal bronchus	10-100 µg/mL DEPs or 2.5-2500 µg/mL suspended PM for 2-48h	Dose dependent ↑ IL-8 and GM-CSF release by DEPs exposure *De novo* protein synthesis	Ohtoshi et al., 1998
	HBEC from bronchial brush biopsies normal subjects	Ultrafine, fine, intermediate and coarse PM at 25-100 µg/mL for 18h	Dose dependent ↑ GM-CSF release to ultrafine/fine PM. No dose-response was detected for the larger PM fractions or carbon particles of the fine sizes. Involvement of MAPK-signaling cascade	Reibman et al., 2002
	HBEC from airway biopsy	50-250 µg/mL coarse, fine and ultrafine PM for 24h	PM-induced ↑ IL-8 release Involvement of TLR2	Becker et al., 2005
	HBEC from lung transplant donors	Baltimore 10-100 µg/mL PM for 1-24h	Dose and time-dependent ↑ IL-6 release to PM Involvement of ROS and NF-κB pathway	Zhao et al., 2008
	HBEC (Lonza)	10 µg/mL PM for 3h	PM-treated HBEC ↑ IL-1α, IL-1β, IL-8 and GM-CSF mRNA levels Role for endotoxin on the particles and ROS production	Wong et al., 2011
	HBEC from brush biopsy	20-80 µg/cm^2 PM_{10} and $PM_{2.5}$ for 4h	Dose-dependent ↑ IL-8 mRNA levels to each particle type Similar responses in BEAS-2B cell line	Silbajoris et al., 2011
	HBEC by brush biopsy	25-100 µg/ mL DEPs for 24h	Dose-dependent ↑ IL-1β and IL-8 protein expression by DEPs exposure Involvement glutathione S-transferases (GSTs) through ROS-associated ERK and Akt activation.	Wu et al., 2012
	HBEC from 5 healthy subjects	500 µg/mL PM_{10} (EHC-93) for 24h	↑ IL-1β production by PM_{10} exposure Involvement NLRP3 inflammasome	Hirota et al., 2012

	HBEC (Lonza)	3 µg/cm² DEPs and fine PM for 6, 18h	Dose dependent DEPs-induced ↑ TSLP mRNA miRNA-375 involved in the TSLP regulation	Bleck et al., 2013
	Air-liquid interface (ALI) culture NHBE (Lonza)	4 treatments of 4h (spaced 48h) with DEPs (SRM1650; 2, 5 and 10 µg/cm²) or coarse / fine / ultrafine PM (1, 5 and 10 µg/cm²)	↑ GM-CSF release after each DEPs/PM treatment that could be maintained up to 5 weeks post exposure. PM had a lower effect than DEPs ↑ IL-6 release after DEPs/PM treatment that could be maintained up to 5 weeks post exposure ↑ IL-1β release at the beginning of the PM treatment	Boublil et al., 2013
	HBEC from bronchial brushings (submerged / ALI)	Coarse, fine and ultrafine PM at 50-250 µg for 4h	↑ IL-6 and IL-8 in submerged PM-exposed HBEC No response in ALI cultures	Ghio et al., 2013
	HBEC from bronchial brushings	10-200 µg/mL DEPs for 18-24h	DEPs-induced dose-dependent ↑ IL-8 mRNA and protein level Involvement of EGFR/autocrine ligands and MAPK pathway	Parnia et al, 2014
	HBEC from brushings	1.1 – 11.1 µg/cm² coarse, fine and ultrafine PM for 24h	Dose-dependent PM-induced ↑IL-8 release Importance particle size and epithelial cell culture	Loxham et al., 2015
	HBEC of bronchial brushings	50 µg/mL PM_{10} or CB for 24h	PM_{10} stimulated ↑ IL-1β, IL-6 and IL-8 mRNA expression. CB is ineffective.	Kumar et al., 2015
	HBEC	100-500 µg/cm³ Urban PM 1649b (NIST) for 24h	Dose-dependent ↑ IL-1β, IL-6 and IL-8 at both mRNA and protein level after PM exposure Involvement of ROS, MAPK pathway and NF-κB	Wang et al., 2017
HBEC ASTHMATICS	HBEC from asthmatic and non-asthmatic subjects	0-100 µg/mL DEPs for 24h	HBEC of asthmatics constitutively ↑ GM-CSF secretion compared to non-asthmatics + DEPs: ↑↑ GM-CSF release in asthmatics	Devalia et al., 1999
	ALI cultures asthmatic and non-asthmatics	100 µg/mL PM (EHC-93) for 24-96h	PM-exposed HBEC from asthmatics have an enhanced ↑ IL-6, IL-8 and GM-CSF release compared to non-asthmatics	Hackett et al., 2011

CO-CULTURE	mDC from blood / HBEC from bronchial brushing normal volunteers	10µg/mL DEPs for 48h	↑ GM-CSF release in the presence of DEP-treated HBEC GM-CSF-induced DC maturation	Bleck et al., 2006
	mDC from blood / HBEC from bronchial brushing normal volunteers	3 µg/cm² DEPs for 4-18h	↑ TSLP mRNA and protein expression in DEPs-exposed HBEC TSLP induced maturation and T cell proliferation of DC	Bleck et al., 2008
	HBEC mDC from blood	3 µg/cm² DEPs for 6-48h	DEP-treated HBEC ↑ IL-33 transcription and protein levels IL-33 induced functional mDC maturation	Bleck et al., 2011
	Co-culture HBEC of bronchial biopsies and blood moDC from control and asthmatic subjects (ALI-culture)	PM for 24h	Monoculture HBEC + PM exposure: ↑ IL-33 production in HBEC from severe asthmatics PM-induced ↓ IL-25 mRNA in control subjects No effect on TSLP Co-culture HBEC + moDC + PM No effect on TSLP, IL-33 or IL-25 (PM impaired TSLP and IL-33 levels that was increased in co-culture of severe asthmatic HBEC)	Gras et al., 2017

↑: cytokine increases in response to DEPs/PM, ↓: cytokine decreases in response to DEPs/PM, ≈: equal cytokine levels

TABLE S2: PARTICULATE MATTER AND TYPE-2 PROMOTING CYTOKINES: FINDINGS IN HUMANS				
Study population		**Material**	**Observations epithelial cytokines**	**Reference**
ADULT	15 healthy volunteers exposed to diluted DE and air for 1h	Bronchial tissue Bronchial wash cells	≈ IL-1β and GM-CSF gene transcript levels after DE exposure compared with air	Salvi et al., 2000
	Intranasal instillation 300µg NIST 1650 DEP both asthmatic and non-asthmatic subjects	Nasal lavage	≈ GM-CSF protein levels post DEP exposure	Kongerud et al., 2006
	87 healthy subjects (office workers vs. traffic-controllers) Monitoring PM_{10}	Exhaled breath condensate (EBC) Nasal lavage fluid (NLF)	≈ IL-1β protein levels in both NLF and EBC between traffic-controllers and office workers	Lima et al., 2013
	Colaus study Monitoring PM_{10} on the day of visit	Blood	Positive association ↑ IL-1β protein levels for every 10µg/m^3 elevation in PM_{10}	Tsai et al., 2012
	40 subjects (20 near highway and 20 urban residents)	Blood	Positive association ↑ IL-1 protein levels with proximity to highways or heavy traffic	Brugge et al., 2013 [471][471][472][473]
	15 healthy subjects exposed to DE and filtered air for 2h	Blood	↑ IL-1β protein levels post DE exposure	Krishnan et al., 2013
	23 healthy adults walking 2 hours along three diverse roadways Mobile PM sampling	Blood	Positive association ↑ IL-1β protein levels with increasing concentrations $PM_{2.5}$	Mirowsky et al., 2015

	72 healthy subjects Monitoring $PM_{2.5}$	Blood	Positive association ↑ IL-1β protein levels with a 10µg/m^3 increase in $PM_{2.5}$	Pope et al., 2016
	85 male subjects (45 taxi drivers vs. 40 office workers)	Blood	↑ IL-1β protein levels in taxi drivers compared to the control group	Barth et al., 2017
PRENATAL LIFE	Birth cohort Monitoring maternal exposure to PM_{10} in the third trimester Proxy traffic-related air pollution (distance to major roads)	Cord blood	Positive association ↑ IL-1β protein levels with PM_{10} GM-CSF expressed only in 2% of samples No strong associations between major road distance and cytokine concentrations	Latzin et al., 2011
	MIREC study Self-reported exposure to street traffic	Cord blood	Positive association Jointly ↑ TSLP and IL-33 protein levels with heavy street traffic	Ashley-Martin et al., 2015
	MIREC study Monitoring Maternal exposure to $PM_{2.5}$	Cord blood	No association of TSLP and IL-33 protein levels with $PM_{2.5}$	Ashley-Martin et al., 2016
CHILDREN	Residential distance to a major road of 577 control and asthmatic children	Blood	↑ IL-1β or IL-33 protein levels in children with asthma compared to controls. No associations of IL-1β or IL-33 protein levels with residential distance to a major road	Rosser et al., 2016
	27 children with asthma and 12 controls (without asthma)	Blood	↑ IL-1β release after ex-vivo stimulation with traffic-related particulate matter for 24h No differences between asthmatics and controls	Negherbon et al., 2017

↑: cytokine increases in response to DEPs/PM, ↓: cytokine decreases in response to DEPs/PM, ≈: equal cytokine levels

TABLE S3: PARTICULATE MATTER AND TYPE-2 PROMOTING CYTOKINES: *IN VIVO* ANIMAL MODELS				
Animal		**Exposure protocol**	**Observations epithelial cytokines**	**Reference**
Acute effects of DEPs/PM	Male A/J and C57BL/6 mice (10-12 weeks)	Intranasal administration 0.25mg/mL DEP in 40µL every other day for 2 weeks	DEP-stimulated ↑ GM-CSF mRNA total lung tissue Role for GM-CSF in AHR, BALF cell recruitment and mucus metaplasia	Ohta et al., 1999
	Sprague-Dawley rats	Intratracheal administration 5-50 mg DEPS (NIST)	↑ IL-1β mRNA in BALF cells day 1 and 30 post exposure with high DEPs levels ≈ GM-CSF mRNA lung tissue	Rao et al., 2005 [479][479][480][481]
	Male ICR mice (6 weeks)	Intratracheal administration 500µg DEPs	DEP-induced ↑ IL-1β protein levels in lung tissue supernatants compared to vechicle	Inoue et al., 2006
	Female BALB/c mice (10-12 weeks)	Intratracheal administration 5-50µg DEPs	≈ BALF IL-1β levels in DEPs and control-exposed groups	Stoeger et al., 2006
	rats	Intratracheal administration 1-5 mg DEPs on day 1	No detection of GM-CSF in BALF	Yokota et al., 2008
	Female C57BL/6 mice (6-8 weeks)	Intratracheal administration 100µg DEPs on day 1, 4 and 7	↑ BALF IL-1β upon DEPs compared to saline Role of IL-1 signalling in DEP-induced inflammation Independent of NLRP3 inflammasome and caspase-1	Provoost et al., 2011
	IRC mice (6 weeks)	Intratracheal administration 2.5-10 mg/kg $PM_{2.5}$ at day 0	Dose-dependent ↑ BALF IL-1β after PM instillation	Park et al., 2011
	Female C57BL/6 mice (6-8 weeks)	Intratracheal administration 200 µg PM_{10} on day 0	PM-induced ↑ BALF IL-1β Role for NLRP3 inflammasome	Hirota et al., 2012
	Male wister rats (8 weeks)	Intratracheal administration 7.5 mg/kg body weight PM_{10} or $PM_{2.5}$ for 14 days	↑ lung IL-1β in the PM groups compared to the controls	Tian et al., 2012
	Male BALB/c mice (7-8 weeks)	Intratracheal administration 100µg PM10 on day 0, 3 and 6	↑ IL-1β in the BALF of PM_{10}-treated mice compared to sham ≈ IL-1β mRNA lung and blood	Farina et al., 2013

	Female C57BL/6 mice (6-8 weeks)	Intranasal administration 200 µg PM_{10} on day 0	PM-induced ↑ IL-1β and GM-CSF in BALF No detection of IL-25, IL-33 and TSLP Role for NLRP3 inflammasome	Hirota et al., 2015
	Male Wistar rats	Intratracheal administration 0.375-24 mg/kg body weight $PM_{2.5}$ on day 1, 3, 5, 7 and 9	PM-induced ↑ IL-1β mRNA and protein level compared to saline exposure	Li et al., 2015
	Male BALB/c mice (8 weeks)	Intratracheal administration 5.5 – 100µg $PM_{2.5}$	↑ IL-1β expression after PM exposure Positive correlation with tissue inflammation and BALF neutrophils	Van Winkle et al., 2015
	Male and female BALB/c mice (6-8 weeks)	Intratracheal administration 4 mg/kg $PM_{2.5}$ for 5 consecutive days	↑ IL-1β mRNA after PM exposure, not on protein level ↑ EGF-EGFR-Akt-NFkB signalling	Jin et al., 2016
	Male BALB/c (6-8 weeks)	Intratracheal administration 0.5 mg $PM_{2.5}$ at day 0 and 2	PM-induced ↑ IL-1β expression at day 7 and release in the BALF at day 3	Wang et al., 2017
	Male Sprague-Dawley rats	Intratracheal administration 40mg/kg $PM_{2.5}$ every other day for 3 tiimes	↑ IL-1β levels in BALF after PM exposure	Liu et al., 2017

Aggravating effects of DEPs/PM	Male ICR mice (6-7 weeks)	Intratracheal administration 100µg DEP every week for 6 weeks - 1µg OVA every 3 weeks for 6 weeks	OVA + DEPs : ↑ GM-CSF protein levels lung compared to vehicle; ≈ GM-CSF levels compared to OVA	Takano et al., 1997
	Male ICR mice (6 weeks)	12h/day, 7 days/week 0.3-3 mg soot/m^3 DE for 40 weeks 10µg OVA IP at week 16 1% OVA aerosol at a 3 week intervals during last 24 weeks	OVA + DE: dose-dependent ↑ GM-CSF protein levels lung compared to vehicle and OVA group	Takano et al., 1998
	Male ICR mice (6 weeks) Male C3H/HeN mice (6 weeks)	1 mg OVA IP at day 7 12 hours/day 2.2 m^3 DE inhalation for 5 weeks + 1% OVA aerosol at week 5	OVA + DE: ↑ GM-CSF protein levels lung compared to DE-saline group; ≈ GM-CSF levels compared to OVA OVA + DE: ↓ GM-CSF secretion in lung supernatants compared to air – OVA	Miyabara et al., 1998 Miyabara et al., 1998 [03]
	Male C3H/He and BALB/c mice (8-10 weeks)	Intratracheal administration 1mg OVA IP at day 7 1x/week DEPs for 5 weeks + 1% OVA aerosol at week 5	OVA + DEPs : ↑ GM-CSF protein levels lung compared to vehicle-saline; ≈ GM-CSF levels compared to OVA	Miyabara et al., 1998 [104]
	Male BALB/c mice (7 weeks)	Intraperitoneal injection 10 µg OVA + 2mg DEPs on day 0.	OVA + DEPs: ↑ GM-CSF and IL-1α in the peritoneal exudate fluid compared to OVA Role for CD8+ T cells in GM-CSF production	Fujimaki et al., 2001
	C57BL/6N and CBA/JN mice (6 weeks)	Intratracheal administration 0-50µg DEPs + 1µg HDM (Derf) four times at 2 week interval	HDM + DEPs: ↑ GM-CSF in lung homogenate compared to HDM Not detected in CBA/JN mice	Sadakane et al., 2002

	Female BALB/c mice (6-8 weeks)	200-2000 µg/m^3 DEPs + 1% OVA aerosol for 10 days	≈ GM-CSF BALF levels compared to OVA and saline	Whitekus et al., 2002
	Female BALB/c mice (6-7 weeks)	20µg OVA IP on day 1 2 mg/m^3 DEPs + 1% OVA inhalation on day 14-17	≈ GM-CSF BALF levels compared to OVA	Hao et al., 2003
	BALB/c, ICR and C3H/He mice (6 weeks)	Intratracheal administration 0-50µg DEPs + 1µg HDM (Derf) four times at 2 week interval	GM-CSF levels not detected in lung homogenate	Ichinose et al., 2004
	Female BALB/c mice (7-8 weeks)	IP 50 µg OVA on day 0 and 14 3 mg/m^3 OVA aerosol for 30min/day, 3 days/week for 4 weeks 50µg PM_{10} or 30 mg/m^3 OVA aerosol on day 49	OVA + DEPs: ↑ IL-33 mRNA and protein BALF levels compared to carbon black; ≈ GM-CSF levels compared to chronic OVA exposure Role for IL-33 in inflammatory cell recruitment	Shadie et al., 2014
	Female C57BL/6 mice (6-8 weeks)	Intranasal administration 25µg DEPs and 1 µg HDM on day 1, 8 and 15	DEPs + HDM: ↑ IL-25 and IL-33 protein lung levels compared to saline, sole DEP or HDM	De Grove et al., 2017

↑: cytokine increases in response to DEPs/PM, ↓: cytokine decreases in response to DEPs/PM, ≈: equal cytokine levels

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Work experience

2012 – present	**PhD candidate in Health Sciences** – Department of respiratory Medicine – Faculty of Medical and Health Sciences – Ghent University – Belgium
2011 – 2012	**Master thesis student** - Department of respiratory Medicine – Faculty of Medical and Health Sciences – Ghent University – Belgium

Education

2013 - 2015	**Teacher Education Program** – Het Perspectief – Ghent - Belgium
2007 – 2012	**Master in Biomedical Sciences** – Major Immunology and infection - Faculty of Medical and Health Sciences – Ghent University – Belgium
2001 – 2007	**Latin and Sciences** – Sint Franciscusinstituut – Melle – Belgium

Additional courses

2013	**Multicolor flow cytometry** – BD Biosciences – Erembodegem – Belgium **Statistical analyses with SPSS** - Ghent University – Belgium **Medical and scientific writing** – Ghent University – Belgium **Basic assistance Training** – Ghent University - Belgium
2012	**Basic ICH Good Clinical Practice Certificate** – Ghent University – Belgium
2011	**Basic Course Laboratory Animal Science (FELASA Cat. C)** – Ghent University Hospital – Belgium

Supervision

2016-2017	Co-promoter bachelor student Medical Sciences (Honour Programme)
2014-2016	Supervision master student Biomedical Sciences

Awards/grants

2016	Travel grant, Faculty Mobility Fund for EMBO conference on innate lymphoid cells, Berlin, Germany, November 30th – December 2nd 2016
	Travel grant, FWO for European Respiratoy Society (ERS) international congress, London, United Kingdom, September 3rd – 7th 2016
2014	Bursary to attend the 12th ERS Lung Science Conference: 'Lung inflammation and immunity', Estoril, Portugal, March 21st – 23rd 2014
2013	Trainee grant, COST training school: 'Functional analyses of chronic lung disease in vivo and in vitro', Munich, Germany, October 7th – 10th 2013

Conferences

- Science Day *(March 2013, Ghent Belgium)*
- IUAP meeting AIReWAY II *(March 2013, Ghent, Belgium)*
- BVP/SPB – GSK Awards in Pneumology *(March 2013, Brussels, Belgium)*
- Cell-VIB symposium: Immunobiology of asthma and allergic diseases *(May 2013, Bruges, Belgium)*
- Functional analyses of chronic lung disease in vivo and in vitro *(October 2013, Munich, Germany)*
- Science Day *(March 2014, Ghent, Belgium)*
- 12th ERS Lung Science Conference *(March 2014, Estoril, Portugal)*
  Poster presentation: Chemerin/ChemR23 pathway in diesel exhaust particles-induced pulmonary inflammation
- ATS international conference *(May 2014, San Diego, USA)*
  Poster presentation: The role of chemerin/ChemR23 axis in diesel exhaust particles-induced pulmonary inflammation
- BVP/SPB – GSK Awards in Pneumology *(June 2014, Brussels, Belgium)*
  Oral presentation: The adjuvant effect of diesel exhaust particles on house dust mite - induced airway inflammation
- IUAP meeting AIReWAY II *(June 2014, Liège, Belgium)*
  Oral presentation: The adjuvant effect of diesel exhaust particles on house dust mite - induced airway inflammation
- Summer school Leuven *(September 2014, Leuven, Belgium)*
  Oral presentation: The activation of group 2 innate lymphoid cells in a mouse model of diesel exhaust particles-enhanced allergic airway inflammation

- EMBO conference on Innate Lymphoid Cells *(September 2014, Paris, France)*
  Poster presentation: The activation of group 2 innate lymphoid cells in a mouse model of diesel exhaust particles-enhanced allergic airway inflammation
- Cell-VIB symposium: Multifaceted roles of type 2 immunity *(December 2014, Bruges, Belgium)*
  Poster presentation: The activation of group 2 innate lymphoid cells in a mouse model of diesel exhaust particles-enhanced allergic airway inflammation
- Science Day *(March 2015, Ghent, Belgium)*
  Oral presentation: Characterization and quantification of innate lymphoid cell subsets in human lung tissue
- ATS international conference *(May 2015, Denver, USA)*
  Poster presentation: Characterization and quantification of innate lymphoid cell subsets in human lung tissue
- BVP/SPB – GSK Awards in Pneumology *(May 2015, Brussels, Belgium)*
  Oral presentation: Characterization and quantification of innate lymphoid cell subsets in human lung tissue
- Studenten onderzoek symposium (SOS) *(May 2015, Ghent, Belgium)*
  Poster presentation: Characterization and quantification of innate lymphoid cell subsets in human lung tissue
- IUAP meeting AIReWAY II *(June 2015, Leuven, Belgium)*
  Poster discussion: Characterization and quantification of innate lymphoid cell subsets in human lung tissue
- Science Day *(March 2016, Ghent, Belgium)*
- GSK/SPB – GSK Awards in Pneumology *(May 2016, Brussels, Belgium)*
  Oral Presentation: Role of type 2 innate lymphoid cells and T helper 2 cells in polluent-enhanced allergic airway inflammation
- IUAP meeting AIReWAY II *(June 2016, Ghent, Belgium)*
  Oral Presentation: Dysregulation of type 2 innate lymphoid cells and Th2 cells impairs pollutant-induced allergic airway responses
- European Respiratory Society (ERS) international congress *(September 2016, London, UK)*
  Poster discussion: Dysregulation of type 2 innate lymphoid cells and Th2 cells impairs pollutant-induced allergic airway responses
- T cell consortium meeting *(October 2016, Rotterdam, The Netherlands)*
  Oral presentation: Innate lymphoid cells type 2 in pollutant-induced allergic airway inflammation
- Netherlands Respiratory Society (NRS) meeting *(October 2016, Utrecht, The Netherlands)*
  Oral presentation: Mechanisms underlying pollutant-induced inflammatory lung responses
- EMBO conference on Innate Lymphoid Cells *(November 2016, Berlin, Germany)*
  Poster presentation: Dysregulation of type 2 innate lymphoid cells and Th2 cells impairs pollutant-induced allergic airway responses
- Science Day *(April 2017, Ghent, Belgium)*
  Oral Presentation: Dysregulation of type 2 innate lymphoid cells and Th2 cells impairs pollutant-induced allergic airway responses

- GSK/SPB – GSK Awards in Pneumology *(May 2017, Brussels, Belgium)*
  Oral Presentation: IL-33 mediated activation of Th2 cells in pollutant-induced allergic airway inflammation

International peer-reviewed publications

- **De Grove KC***, Provoost S*, Verhamme FM, Bracke KR, Joos GF, Maes T and Brusselle GG. Characterization and quantification of innate lymphoid cell subsets in human lung. Plos One. 2016; 11(1):e0145961. (* equal contribution)
  IF: 2.806, ranking in multidisciplinary sciences: 15/64

- Provoost S*, **De Grove KC***, Fraser GL, Lannoy VJ, Tournoy KG, Brusselle GG, Maes T, Joos GF. Pro- and Anti-Inflammatory Role of ChemR23 Signaling in Pollutant-Induced Inflammatory Lung Responses. Journal of Immunology. 2016; 196(4):1882-90. **(*Equal contribution)**
  IF: 4.856, ranking immunology: 34/151

- Kumar S, Lanckacker E, Dentener M, Bracke K, Provoost S, **De Grove KC**, Brusselle GG, Wouters E, Maes T, Joos GF. Aggravation of Allergic Airway Inflammation by Cigarette Smoke in Mice Is CD44-Dependent. PLoS One. 2016 Mar 21; 11(3).
  IF: 2.806, ranking in multidisciplinary sciences: 15/64

- **De Grove KC***, Provoost S*, Hendriks RW, McKenzie ANJ, Seys JM, Kumar S, Maes T, Brusselle GG, Joos GF. Dysregulation of type 2 innate lymphoid cells and Th2 cells impairs pollutant-induced allergic airway responses. Journal of Allergy and Clinical Immunology. 2017; 139(1):246-257. (*Equal contribution)
  IF: 13.081; ranking allergy: 1/26

- Teufelberger AR, Nordengrün M, Braun H, Maes T, **De Grove KC** , Holtappels G, O'Brien C, Provoost S, Hammad H, Gonçalves A, Beyaert R, Declercq W, Vandenabeele P, Krysko DV, Bröker BM, Bachert C, Krysko O. The IL-33/ST2 axis is crucial in type 2 airway responses induced by the Staphylococcus aureus protease SplD. Journal of Allergy and Clinical Immunology. 2017; 139(1):246-257.
  IF: 13.081; ranking allergy: 1/26

- **De Grove KC**, Provoost S, Brusselle GG, Joos GF, Maes T. Insights in particulate matter-induced allergic airway inflammation: focus on the epithelium. Clinical and experimental allergy. 2018.
  IF: 5.264, ranking allergy: 4/26.

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